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THE EXPRESSION OF ANGIOGENIC GROWTH FACTORS AND EXTRACELLULAR MATRIX PROTEINS IN CENTRAL NERVOUS SYSTEM VASCULAR MALFORMATIONS

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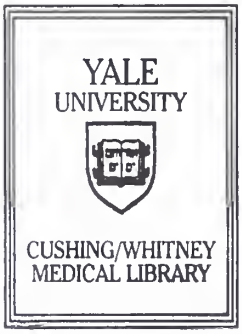
1996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The Expression of Angiogenic Growth Factors and Extracellular Matrix Proteins in Central Nervous System Vascular Malformations

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

By
Jiyon Lee, B.S.
1996

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ABSTRACT

THE EXPRESSION OF ANGIOGENIC GROWTH FACTORS AND EXTRACELLULAR MATRIX PROTEINS IN CENTRAL NERVOUS SYSTEM VASCULAR MALFORMATIONS.

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Arteriovenous malformations (AVM) and cavernous malformations (CM) are two clinically significant types of vascular malformations of the central nervous system. Though both have been extensively described in the neurosurgical literature with regard to their clinical and biological behavior, diagnosing imaging characteristics, and surgical considerations, very little investigative effort has been directed at exploring the molecular pathogenesis and evolution of these lesions. The possible role of angiogenic growth factors in conjunction with extracellular matrix proteins in their genesis has not previously been considered. In this study, we subject twelve neurosurgical specimens (7 AVMs, 5 CMs) to a battery of immunostaining for two known angiogenic growth factors: vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF); and selected matrix proteins: laminin (LN), fibronectin (FN), collagen type IV (CoIV), and alpha smooth muscle actin (ASMA). VEGF was expressed by all twelve lesions, localized primarily in the subendothelium and perivascular tissue. Basic FGF was faintly expressed in four of seven AVMs and four of five CMs in the media of AVM vessels, and the subendothelial layer and intercavernous matrix of CMs. The pattern of angiogenic growth factor expression was correlated with the expression of matrix proteins. LN was not expressed in any of the CMs, confirming previous reports from our laboratory. However, FN was more prominent in CMs. CoIV and ASMA expression occurred in every lesion. We conclude that angiogenic growth factors are expressed in both AVMs and CMs on the CNS. The pattern of immunostaining suggests diffuse activation of angiogenesis without specific relation to individual vessel types or recent clinical hemorrhage while the pattern of matrix proteins expression suggests that CMs lack mature vessel wall elements and have an "angioproliferative" matrix milieu as compared to AVMs. We hypothesize that AVMs and CMs may represent "dysvasculogenesis" and "dysangiogenesis" respectively, both concepts discussed in depth in this thesis. Clarifying the role of angiogenesis in vascular malformations may provide insight into their pathogenesis and suggest novel strategies for modification of their behavior.

ACKNOWLEDGEMENTS

I wish to thank Dr. Issam Awad for providing the clinical concepts for this thesis.
To my advisors, Dr. Awad and Dr. Gregory Criscuolo, for their continuing professional guidance.
To Dr. Jung Kim in Neuropathology, for teaching me the ropes in the lab and for his consistently good-humoured kindness.
To my loving parents, who sacrificed so much to give me a better life, who taught me ambition at an early age but then (fortunately) mellowed with time.
To my three brothers, Du Hee, Steven, and David, for their encouragement and loving support.
To Mr. and Mrs. David Bliss, for "adopting" me with such warm generosity.
Last but not least, to Jamie, the man who saw me through it all, and is not afraid.
Thank you, Lord, for all the blessings that you have given us.

TABLE OF CONTENTS

I. INTRODUCTION & BACKGROUND

A. Vascular Malformations in the Central Nervous System

Among the various pathological entities that may affect the central nervous system is the category of lesions called vascular malformations. Unlike vascular tumors, such as hemangioblastomas, vascular malformations are anomalous collections of non-neoplastic blood vessels. They can occur anywhere along the neuraxis (brain and spinal cord). Based on distinct clinical, radiologic, and pathologic characteristics, there are four widely accepted classifications of vascular malformations described in the neurosurgical literature. These include venous, capillary, arteriovenous (AVM), and cavernous malformations (CM), names which arose from the gross and microscopic pathological appearance (Russell & Rubinstein, 1977; McCormick & Boulter, 1966) of these lesions. A recently recognized fifth type is called the "mixed" vascular malformation (Awad et al., 1993)(1), the majority of which have elements of cavernous and either AVM or capillary telangiectasia within the same lesion.

The most benign is the capillary telengiectasia, a cluster of small vessels found within normal brain tissue (fig. 1, page 4) The vast majority are clinically silent and are found at autopsy where they appear as pink blushes (19). They are often "occult" (not detectable) on computed tomography (CT), magnetic resonance imaging (MRI), and angiography.

Venous malformations (also called venous angioma) are collections of enlarged veins, histologically exhibiting typical venous walls and usually clustered around one or more larger draining veins (fig. 2, page 4). They are little more than varices and pose no great clinical danger as they generally drain normal brain parenchyma. Surgical resection is usually not indicated, and indeed may be unwise for normal vascular drainage to occur. They typically appear as a "caput medusa" on CT, MRI, and angiography.

Far greater clinical impact is made by AVMs and cavernous malformations, the two subtypes of cerebrovascular malformations of particular interest in this study. AVMs are clusters of abnormal arteries and veins that form a direct high-pressure shunt due to the congenital absence of intervening capillary bed (fig. 3, page 5). Grossly, AVMs have been described as a "snarl," or mass of tangled vessels (19). Vessel walls contain elastin and smooth muscle and may be separated by intervening brain parenchyma. These high flow lesions demonstrate the ability to "recruit" neighboring ("en passage") arteries (5- 7) and contain

both hypertrophied (thickened) arteries and "arterialized" veins, all elements which contribute to their aggressive growth and propensity for sudden hemorrhage. For diagnosis, computed tomography (CT) is moderately sensitive and specific for AVMs and magnetic resonance imaging (MRI) has higher sensitivity but moderate specificity. On angiography, AVMs show arteriovenous shunting but on occasion may be angiographically-occult.

It is not uncommon for these congenital lesions to remain undetected until they present in the second to fifth decades as a sudden fatal hemorrhage. Other presentations include headache, seizures, or focal neurological deficit. In infancy and childhood, these malformations can occur in the great vein of Galen and cardiac failure may be the initial presentation. Cerebral AVMs can exist as part of clinical syndrome called Hemorrhagic Hereditary Telengiectasia (HHT) or Osler Weber Rendu disease.

Cavernous malformations (or cavernous angioma) are quite distinct from AVMs in several aspects (1-5, 8-17, 19). Histologically, CMs are clusters of dilated vessel spaces with no intervening brain parenchyma (fig. 4, page 5). The vessels are thin walled with simple endothelium (12) and a thin fibrous adventitia, devoid of obvious smooth muscle layers and elastin, elements which may help explain their sluggish flow pattern (13) and tendency to thrombose. These lesions may grow by repeated intralesional hemorrhages and "reactive" vascular proliferation in the area of the lesion; the walls may become thickened, calcified, and even ossified from chronic damage. Compared to AVMs, CMs grow less aggressively. The frequent presence of capillary telangiectasias in the periphery of CMs has given rise to speculation that they may be a "baby CMs" and that these two vascular lesions are in fact part of a continuum (10).

CMs usually present with seizures, focal neurologic deficit, or gross intracranial hemorrhage. Episodes of hemorrhage tend to be recurrent, though usually self-limited because of low driving pressures (13). Surgical treatment is warranted in cases where morbidity will be improved, but is not performed electively for lesions that are clinically silent (14). These lesions may recur after surgery. For diagnosis, the MRI is the superior imaging modality for CMs, demonstrating higher sensitivity and specificity than the CT. On angiography, CMs are occult, showing a faint capillary blush at best. Overall incidence is estimated at 0.4%; 10-30% of those patients may have the autosomal dominant familial form of the disease, which has

to grow, and also may arise de novo (17). The familial gene for CMs has been identified on chromosome 7 (16) and has been successfully mapped at this institution (15).

These two vascular malformations have been extensively described in the neurosurgical literature with respect to their clinical significance, biological behavior, imaging characteristics, and surgical considerations. However, far less investigative effort has been directed at exploring the pathogenesis and evolution of these lesions. Given the dynamic growth of these vascular malformations (5- 10, 12-14, 17), as documented by imaging over time, our fundamental presumption is clear: they are vascular lesions that appear to grow in the context of and possibly in reaction to the surrounding brain parenchyma. In contrast to vascular tumors (e.g., hemangioblastoma) which grow by the uncontrolled replication of endothelial cells that invade surrounding tissue and form disorganized vessel structures, CMs and AVMs appear to have controlled vascular proliferation that becomes a functional part of the growing lesion. In light of this distinction, and in recognition of the tremendous research in angiogenesis in the past decade, this study was developed to elucidate the possible role of angiogenesis growth factors in the evolution of cerebrovascular malformations.

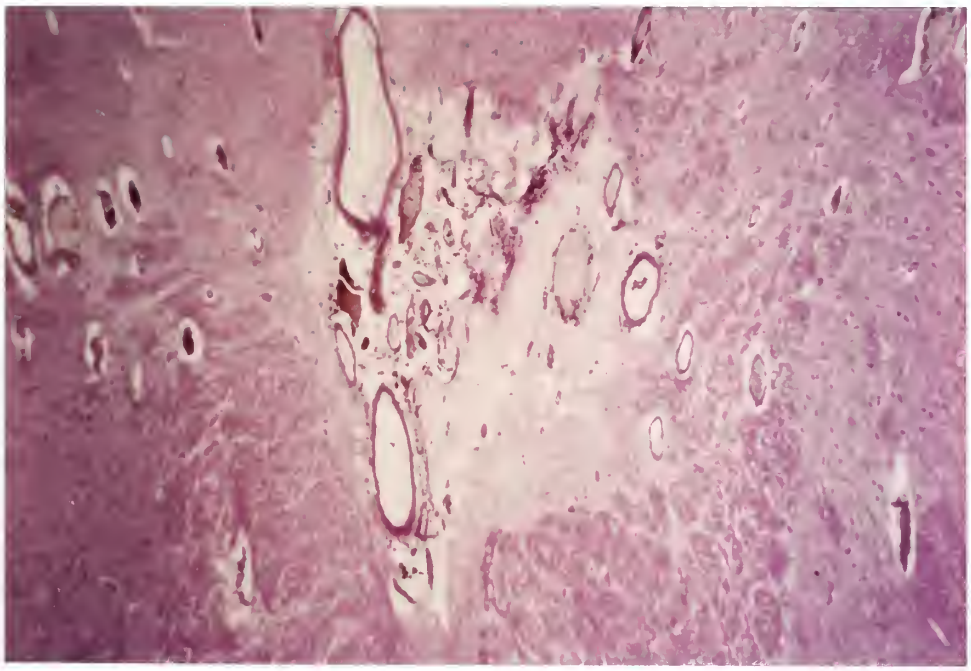


Figure 1. Capillary telangiectasia found incidentally in the pons at autopsy. Notice the cluster of dilated capillaries in the middle lower field. (H&E 4X)(Photo JYL)

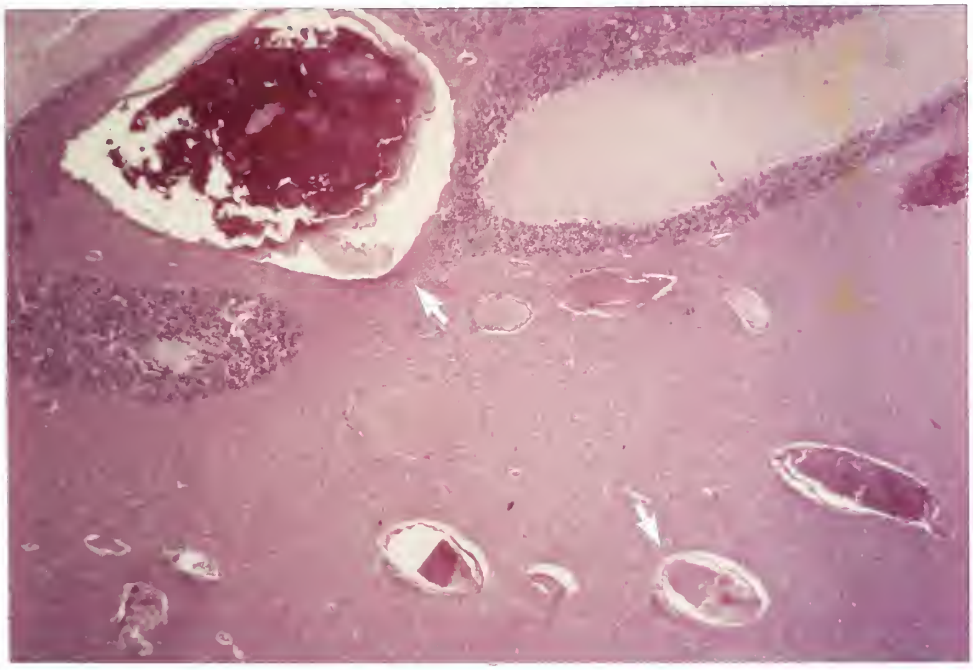


Figure 2. Venous angioma (venous malformation) in cortical white matter, also found at autopsy. There is large draining vein in the upper left field and several lesser enlarged veins below. (H&E 2X)(Photo JYL)

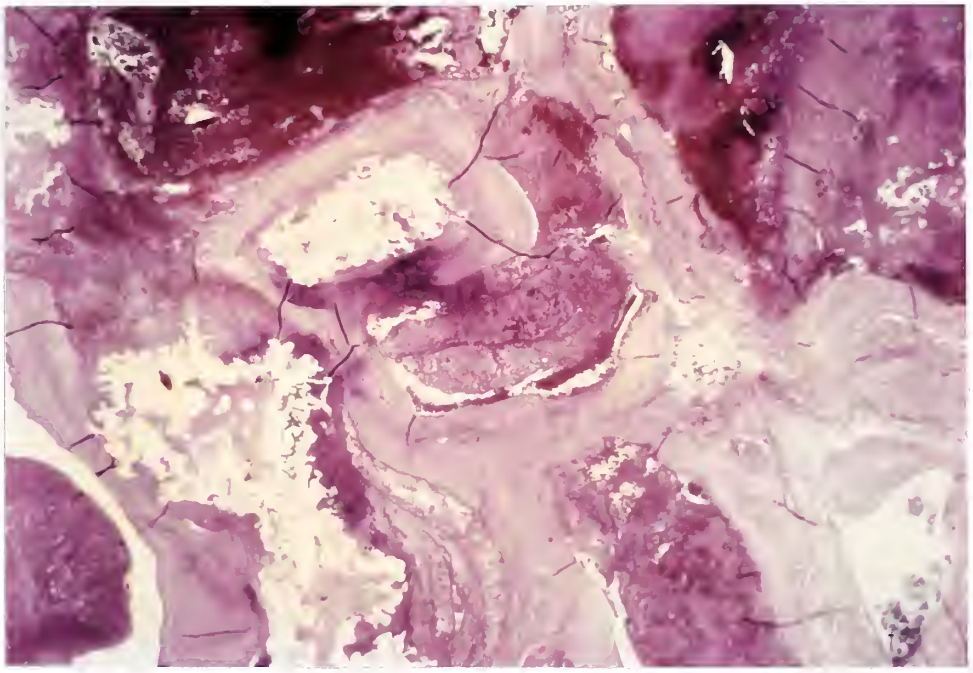


Figure 3. Arteriovenous malformation showing mixture of arteries and venous structures of varying sizes with intervening brain parenchyma. (H&E 2X)(Photo JYL)

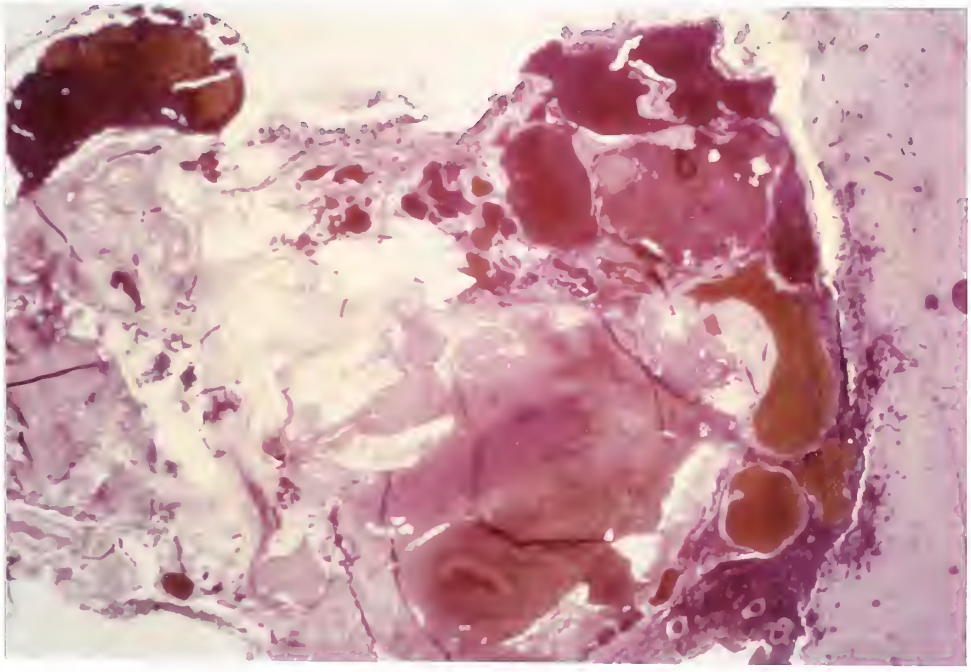


Figure 4. Cavernous malformation (cavernous angioma) contains enlarged, thin walled vascular spaces of varying sizes and shapes. This one is adjacent to brain parenchyma but notice absence of intervening brain parenchyma between the caverns. (H&E 2X)(Photo JYL)

B. Angiogenesis & Angiogenic Growth Factors

In embryonic development, blood islands (hemangioblasts) in the yolk sac separate from the splanchnopleuric mesoderm and organize into loosely associated cords that form the preliminary vascular network (Sabin, 1920; Romanoff, 1960; Wagner, 1980; Pardanaud, 1987)(21, 22). It was presumed that the vasculature of the embryo originated from the invasion of some of these hemangioblasts from the yolk sac. However, Reagan (1915) showed that embryonic capillaries formed even if the connection between extra- and intraembryonic tissues were eliminated. A more recent illustration that at least two modes of origin of embryonic vessels existed was provided in vivo in the Japanese quail embryo. Using monoclonal antibody to label the "presumptive endothelial cells" (PECs) at the 1-somite stage, Coffin & Poole (1988) showed that in situ aggregation of PECs formed the dorsal aorta and cardinal veins, but the intersomitic arteries, vertebral arteries, and cephalic vasculature arise from sprouting of the rudimentary vessels (21). Risau & Lemmon (1987) proposed the term "vasculogenesis" to refer to the in situ aggregation and differentiation of endothelial cells (fig. 5, page 18), to distinguish it from "angiogenesis," which has been refined to mean the sprouting of new vessels from preexisting vessels (22).

The process of angiogenesis is a multistep one that occurs mainly from the sprouting of small post-capillary venules (Ausprunk & Folkman, 1977). Local degradation of the basement membrane precedes the migration of endothelial cells (ECs) toward an angiogenic stimulus. These ECs align to form a solid sprout and a lumen is created by the curvature formed within each cell. The new vessel is lengthened by cellular proliferation and strengthened by pericytes and the basement membrane that the ECs produce. Blood flow starts when two hollow sprouts form a loop and the next generation of sprouts originate from the apex of the loop (Ausprunk & Folkman, 1977).

Angiogenesis occurs in numerous conditions such as organogenesis (Ekblom et al., 1982; Risau & Ekblom, 1986; Risau, 1986), the inflammatory process and normal wound healing (Cliff, 1963; Clark et al., 1982)(23, 24), and cyclically in the corpus luteum (Bassett, 1943). In these situations, angiogenesis plays a major but controlled physiological role in growth and repair. However, angiogenesis is involved in many pathological states as well and is indeed the major facilitator of the clinical problems associated with diabetic retinopathy (Malecaze, 1994), atherosclerosis and tumor growth (25- 27). In contrast to embryonic

are in a proliferative mode, endothelial cell turnover in adult capillaries is normally infrequent, measurable in years (Denekamp, 1984; Engerman et al., 1967). The intriguing question of how angiogenesis is so well-regulated in some situations and dysregulated in others became the puzzle to solve in these past two decades.

It is in the area of tumor biology that angiogenesis has been studied with the most clinical interest. Folkman et al. (1963) observed that tumors implanted into isolated perfused organs did not grow beyond a few mm in diameter yet grew rapidly beyond 1 cm^3 and killed their hosts when reimplanted into mice. Closer inspection found that in vivo, the tumors became vascularized but in the isolated organs, they did not. In fact, capillary endothelium gradually degenerated with ongoing perfusion (Gimbrone et al., 1969) and therefore, could not support tumor growth. Experiments by Gimbrone et al. (1972) indicated that tumors separated from their vasculature stopped growing at 1-2mm^3 but quickly grew when vessel supply was restored. An interesting histological observation was made by Tannock (1968) when he noticed tumor cells surrounded capillaries at distances between 150 and 200um, which is the diffusion distance of oxygen. DNA synthesis decreased with increasing distance of tumor cells from the nearest open capillary. These observations further supported the theory that tumor growth was angiogenesis-dependent. However, whether the tumor cells directly induced neovascularization or caused it indirectly , e.g. as part of an inflammatory response, was not clear until the isolation of diffusible angiogenic factors in the 1970's from tumors and conditioned media of transformed cells (Folkman et al., 1971; Klagsbrun et al., 1976).

Closer study of angiogenesis and angiogenic factors was facilitated by the development of several new experimental models in the 1970's (25). The ease of endothelial cell cultures from human umbilical vein, aorta, and capillaries allowed in vitro isolation and testing of candidate growth factors. The chick embryo chorioallantoic membrane (CAM) was used to test angiogenic activity of tumor extracts (Klagsbrun et al., 1976; Ausprunk et al., 1974). An in vivo model using the normally avascular cornea of animals was developed to detect vessel growth toward an implanted tumor or angiogenic factor (Gimbrone et al., 1974). The advent of biodegradable polymers made possible the sustained release of angiogenic factors in in vivo experiments (Langer & Folkman, 1976).

Among the first angiogenic growth factors to be isolated were basic fibroblast growth factor (bFGF) from bovine brain and pituitary (Gospodarowicz, 1978) and acidic growth factor (aFGF) from hypothalamus (Maciag et al., 1979). The serendipitous discovery in 1983 that an endothelial growth factor (ECGF) from rat chondrosarcoma had marked affinity to heparin was crucial in facilitating its purification via heparin-affinity chromatography. This homogenous cationic protein was found to be angiogenic in both the rat cornea and chick embryo bioassays (Shing et al., 1985) and later identified as aFGF.

The use of heparin-affinity chromatography achieved the purification of many endothelial growth factors and by 1985, the protoypes of two classes of angiogenic heparin binding proteins had been identified: basic FGF, a 146-amino acid polypeptide, and acidic FGF, a 140-amino acid polypeptide. By 1986, the primary amino acid sequences were determined (Esch et al., 1985), and the genes for bFGF (Jaye et al., 1986) and ECGF (precursor to aFGF)(Mergia et al., 1986) were cloned from libraries of complementary DNA.

1. Basic Fibroblast Growth Factor

Basic and acidic fibroblast growth factors (FGFs) are related molecules that show 55% sequence homology and have similar biological roles in regulating cellular growth (29, 30). Basic FGF was initially identified for its ability to induce the phenotypic transformation and proliferation of BABL/c 3T3 fibroblasts (Gospodarowicz et al., 1974); aFGF for its ability to cause proliferation and delayed differentiation of myoblasts (Gospodarowicz et al., 1975) and later for its activity as an endothelial cell mitogen (Lemmon et al., 1982; Maciag et al., 1979). Unlike aFGF, which has only been localized in the brain and retina, bFGF has been purified from a wide variety of tissues, both mesodermal and neuroectodermal in origin. These include brain, pituitary, adrenal (Gospodarowicz et al., 1986), retina, kidney (Baird et al., 1985), placenta (Moscatelli et al., 1986)(31), corpus luteum (Gospodarowicz, 1985), prostate, and thymus. In vitro evidence that bFGF but not aFGF is produced by capillary endothelial (32) and VSMC (33) may very well explain the prevalence of bFGF versus aFGF (29), especially in well-vascularized organs, as well as provide a mechanism by which bFGF works in both an autocrine and paracrine fashion to promote EC activity (32-33). Subsequent in situ hybridization studies indicate that

bioactive bFGF is produced in other normal cell populations such as corneal EC, lens epithelial cells, adrenal cortex, granulosa cells, myoblasts, osteoblasts (29) and macrophages (34).

Both cationic (bFGF) and anionic (aFGF) classes of FGFs are not only mitogenic for ECs in vitro, they are angiogenic in vitro (35) and in vivo (35-36), as seen by the chick CAM and cornea bioassays (25). They are also capable of inciting richly vascular granulation tissue in sponges implanted subcutaneously in the rat (25), though aFGF is 30-100 times less potent than bFGF (Bohlen et al., 1985)(43). The greater interest in bFGF as a widespread angiogenic factor resulted in numerous studies heralding its upregulated presence in many tumors, including melanoma, hepatoma, chondrosarcoma (29), and brain tumors. It is this last category that is of special relevance to this study. In vitro evidence of bFGF production from glioma (primary brain tumor) cells in culture (37- 38) were followed by immunohistochemical studies showing the presence of bFGF in human glioma sections (39-41), in patterns that suggest its involvement in the malignant progression of gliomas. It was noted that bFGF staining occurred in the cytoplasm of glioma cells (39-41); endothelial cells (39-41), especially those closer to the tumor (41); fibroblasts from connective tissue stroma from papillomas, and metastic tumor cells in some studies (39) but not others (40). Meningiomas, which, like malignant gliomas, are noteworthy for being highly vascular, had the highest percentage of positive staining cells (39). Researchers further confirmed these findings with in situ hybridization studies that showed the presence of bFGF mRNA in tumor and endothelial cells in the majority of gliomas and meningiomas tested but none in nontumorous brain tissue controls (40).

Receptors that bind FGFs with high affinity and high specificity exist on ECs (Friesel et al., 1986), VSMC (Winkles et al., 1987), myoblasts (45), and fibroblasts. They bind bFGF with much higher affinity than they bind aFGF (Huang & Huang, 1986)(43). In addition to its mitogenic properties, bFGF has profound effects on EC collagen, fibronectin, and proteoglycans production (Tsueng et al., 1982; Godpodarowicz, 1983). It has been shown that monolayers of quiescent capillary ECs produce basement membrane rich in collagen type IV (which exists strictly in basement membranes) but migrating or sprouting ECs, e.g. those exposed to bFGF, switch to collagen types I and III (Madri & Williams, 1983).

Though the evidence of bFGF's involvement in several components of angiogenesis is compelling, its mechanism of action is still unclear. To begin with, upon sequencing the growth factor, it was quickly

noticed to lack the conventional signal sequence that would direct their release via the normal secretory pathway (42). Yet numerous cell populations have specific FGF-Rs on their surface (43-45), indicating that bFGF reaches the exterior of the cell somehow. So it was theorized that sublethal injury or necrosis of bFGF producing cells was the method by which its action could be released, which is reasonable considering the situations in which a growth factor both angiogenic and mitogenic for fibroblasts and endothelial cells would be efficacious. Subsequent researchers localized bFGF to the Descemet basement membrane of the bovine cornea (46), providing the first in vivo evidence that bFGF may be sequestered in the basement membrane in normal tissues. It is speculated that bFGF is bound to heparin sulfate proteoglycans and secreted into the extracellular matrix , where it is stored (46) and protected from degradation (32-33). Folkman et al. (46) and Vlodasky et al. (47) theorize that by storage in the ECM, angiogenesis can be prevented until injury to the BM causes sustained release of bFGF. Once released, endogenous bFGF can begin the angiogenic process by local degradation of the ECM via stimulation of collagenase activity, plasminogen activator and BM proteases production (27, 51), and metalloproteinase production (48). Then, bFGF can induce EC migration, cell invasion (48), and cellular proliferation, which are also components of angiogenesis. In addition to storage of bFGF until the need arises, it is possible that another mechanism of regulation is by controlling the number of receptors for bFGF available (48) in response to other stimulit through mechanisms that are not clearly determined.

2. Vascular Permeability Factor/ Vascular Endothelial Growth Factor

Vascular permeability factor (VPF) was originally discovered as the protein responsible for the increased microvascular permeability and ascites production in rodent tumor cell lines (49). Further investigation showed that this 34-42kD protein was produced from multiple tumor cell lines including the guinea pig line 10 (bile duct carcinoma), human bladder, cervical, fibrosarcoma, and osteogenic sarcoma (49). Matched pairs of tumor-nontumor cell lines showed that tumor lines had 14 fold greater VPF concentration, suggesting a role in tumor biology (49). Testing with the Miles assay on guinea pig skin showed that not only did the purified protein cause a rapid and reversible increase in microvascular permeability, this activity could be blocked upwards of 90% by the addition of anti-VPF antibodies (made from gp line 10 immunization) (49). The absence of accompanying inflammatory reaction or mast cell

degranulation in the guinea pig skin ruled out inflammatory mediators such as histamine and prostaglandins as the mechanism of increased permeability.

During this time in 1989, Ferrara et al. (50) and Gospodarowicz et al. (51) independently noticed that the media from pituitary folliculostellate cell cultures was strongly mitogenic for endothelial cells. Until then, bFGF had been the best characterized EC-mitogen. Detecting little to no bFGF in the media (consistent with its lack of signal sequence), they theorized that some other EC-specific growth factor was present. Researchers set about isolating this new protein with the use of heparan sepharose affinity chromatography and high performance liquid chromatography (50-51), purifying the protein 1000 fold. They tested this protein on endothelial cells from various regions, found it was consistently mitogenic and strictly for ECs. Ferrara et al called this protein "vascular endothelial growth factor (VEGF)."

Multiple characteristics indicated that this was a previously undiscovered protein. The NH2 terminal sequence (52) and physical properties did not match those of other known GFs, e.g. ECGF, transforming growth factor beta (TGFb), transforming growth factor alpha (TGFa), platelet derived growth factor (PDGF), keratinocyte GF (51). Acidic and basic FGF were unlikely candidates given their wide target cell range and were firmly ruled out by lack of absorption with neutralizing polyclonal antibodies and lack of cross reactivity in radioimmunoassays specific for FGFs (51). Though similar to the newly identified platelet-derived endothelial cell growth factor (PD-ECGF) in target cell specificity and molecular weight, VEGF had a different secondary structure (dimer, not single polypeptide), a secretion signal, and a 10-20 fold greater potency than platelet derived endothelial cell growth factor (PD-ECGF) (51).

Several researchers began to suspect that VPF and VEGF may be the same protein, including Connolly et al. (53) who conducted a series of compelling in vitro and in vivo experiments to test this possibility. They used purified gp VPF and found that it clearly was mitogenic for endothelial cells in culture and, as expected, lost greater than 95% mitogenicity when blocked with polyclonal antibodies to its NH2 terminal sequence (52-53). They then used two animal models to test its angiogenic capabilities. In the rabbit bone assay, purified VPF was infused via osmotic pumps into healing mandibular bonegrafts for 14 days. Histological sections clearly showed more vessels present on the VPF infused side, compared to the opposite non-infused site, especially near the outlet of tubing from the pump. In the second model used,

the normally avascular rat cornea showed an angiogenic response at even low doses of VPF. Both of these angiogenic responses were blocked using antibodies, confirming the action of VPF. Through their experiments and others', Connolly et al. concluded that highly purified VPF was identical to VEGF in molecular and physical properties, mitogenic and angiogenic performances in vitro and in vivo, and inactivation by specific antibodies. Subsequent cDNA cloning studies confirmed that they are the same molecular species (54-55).

The combination of VPF/VEGF's two potent actions is of special clinical significance in the setting of brain tumors. Within the confines of the unyielding cranial vault, a tumor that is surrounded by an accompanying edema presents the epecially worrisome threat of brain herniation and sudden death that tumors in other body cavities do not. In general, those brain tumors that frequently cause edema are the malignant gliomas and metastases, and for decades, dexamethasone (corticosteroid) has been empirically given to decrease this edema in neurosurgical patients. To approach the intriguing question of how this vasogenic edema occurs, Bruce et al. (1987) searched for a vascular permeability factor in the conditioned media of various brain tumor cultures, including malignant gliomas, astrocytomas, and meningiomas. Using heparan-sepharose affinity techniques to purify and the established Miles assay to test the candidate protein, it was found that bioactive VPF was in fact produced by tumor cells, but only those of the malignant glioblastoma (GBM)(56). Others have produced immunohistochemical (57-59) and in situ hybridization studies (60) that correlate VEGF detection with edema presence, though VEGF expression may correlate better with the vascularity of the tumor than its edema production (60).

Criscuolo et al. (1988) conducted the next set of experiments to further characterize VPF and begin to gather evidence that VPF works directly on the EC and not via another intermediate effector, such as prostaglandins (61). Using chemical cross linking experiments, several researchers had identified specific high affinity binding sites on the EC membrane (61-63, 67). Building on the previous experiment that showed the Miles assay could be blocked by a 2 hour pretreatment with dexamethasone (56), they found that the steroid induced inhibition of VPF action was partially reversed by pretreatment with actinomycin D (transcription inhibitor). This result indicated that dexamethasone inhibition of VPF works by the induction of de novo protein synthesis (61).

Criscuolo et al. (1989) next used VPF isolated from human glioma cells (HG-VPF) and ECs preloaded with fluorescent intracellular calcium probes to demonstrate a rapid, transient, reliable calcium influx upon exposure to VPF. Dexamethasone suppressed the calcium influx. Furthermore, this response was inhibited by EGTA (calcium chelating agent) and nonspecific channel blockers, e.g. Li^{++}, Co, Nm, La, but not inhibited by verapamil (voltage-gated channel blocker). This effect was seen in EC cultures from a variety of tissues (brain, lung, adrenal) and from various species (human, rat, bovine)(64).

Ongoing molecular studies further characterized the VEGF protein, showing its structural similarity to PDGFb chain (54), the existence of multiple isoforms (53-55) of MW 121, 165, 189, and 206, which are achieved by differential mRNA splicing (Houck et al., 1991)(51), and even the presence of potential high affinity binding sites on the EC membranes of a wide variety of normal tissues such as the brain, kidney, and the corpus luteum (63). With the accumulating evidence that VEGF may play an active role both in the angiogenesis in pathological states, as well as the maintenance of normal vasculature in tonic tissues, the next questions to explore became which components of angiogenesis do VEGF control and how?

Within the past year have come startling new experiments that suggest different VEGF receptors are involved in different stages of vasculogenesis (66) and angiogenesis (65-67, 69-71). Using a technique of homologous recombination in embryonic stem cells in mice, researchers found that when made deficient in the VEGF receptor named Flk-1, the mice died from failure to form blood islands and deficient vasculogenesis (69). In a related experiment, mice deficient in Flt-1 receptors also died in utero, but not from lack of vascular cell differentiation for there appeared to be ECs in both extra- and intraembryonic tissues. Rather in these mice, abnormal vascular channels were formed, suggesting that this receptor is involved in proper EC-EC interaction or cell-matrix interactions during development (70). A third related study showed that mice embryos with a mutation in either the Tie-1 receptor or the Tie-2 receptor genes also died, of still different abnormalities. The Tie-1 deficient mice were found to have normal vessel pattern but ultrastructurally abnormal vessel integrity leading to edema and hemorrhage. The Tie-2 deficient mice showed a very abnormal vessel pattern, with uniformly dilated vessels and a remarkable absence of capillary sprouts. Taken together, these data demonstrate that different VEGF-R's activate

different cellular responses and that multiple signalling pathways are necessary to fully execute EC differentiation and vascularization.

C. Extracellular Matrix & Basement Membrane

To review the traditional teaching of arterial vessel architecture, the endothelium is the single layer of endothelial cells immediately adjacent and exposed to the lumen. The endothelium along with its thin layer of subendothelial connective tissue (basement membrane) are together called the tunica intima. This layer thickens with age and is the site of atherosclerotic injury. The endothelium is surrounded by a layer of myointimal cells, which play an important role in regulating cerebral blood flow. The middle layer (tunica media) is broad and composed of many sheets of smooth muscle cells, some elastin and collagen; this layer forms most of the thickness of an arterial wall and is the site of morphological changes seen in hypertension. In muscular arteries, elastin is mostly concentrated in the internal elastic lamina (IEL) immediately external to the intima, and in the adventitia. Finally, the outermost layer (tunica adventitia) is made of loose connective tissue that blends into the surrounding connective tissue and contains the vasovasorum and nerves that serve the metabolic needs of the vessel wall (26, 74). In veins and capillaries, the main differences lie in the media and adventitial layers. Veins do have some layers of smooth muscle cells, though they make up a small proportion of the thickness of the wall; the adventitia is relatively thicker than in arteries. In capillaries, the adventitia is ill-defined and the media is completely absent. A population of cells, called pericytes, serve the contractile function for the smaller caliber vessels. They form a layer adjacent to the endothelium that exists throughout the transition from arterioles to venules (76).

The basement membrane (BM) is a specialized portion of the extracellular matrix (ECM) that lies adjacent to the endothelial cells of the entire vascular system, and to all epithelial, smooth muscle, and skeletal muscle cells of the body. Not only is the basement membrane adherent to these cells, it is actually produced by these cells. During neovascularization, for example, initial capillary endothelium does not exhibit a basement membrane. As vessels mature, cells flatten and produce extracellular matrix proteins that eventually become a continuous (except in tissues such as liver) and stable BM (77). In addition to providing structural support, BMs also play a variety of other important roles (78) such as anchoring and

maintaining polarity of cells, influencing differentiation, and possibly even serving as storage site for substances such as growth factors (46-47). The rest of the extracellular matrix is crucial for cellular migration and adhesion (75), differentiation, and proliferation (80-82), all functions involved in the angiogenic process.

Electron microscopic (EM) studies have illustrated two to three layers to the basement membrane. The basal lamina (BL) is electron dense under EM but under light microscopy, it is the composite of all layers that is recognized as the basement membrane. The BM is composed of continuous mats of specialized ECM that underlie all endothelial and epithelial cells, as well as surround individual myocytes, lipocytes, and schwann cells, to separate cells from the surrounding ECM. The BL is synthesized by cells resting on it and is comprised mostly of tough mats of collagen.

The macromolecular components of the BM are of two main classes: the glycosaminoglycans (GAGs), and the fibrous proteins that serve as adhesion molecules, e.g. laminin (LN) and fibronectin (FN)(Hynes & Yamada, 1982; Ruoslahti et al., 1982; Timpl et al., 1983; Kleinman et al., 1985)(75), and those that serve as structural proteins, e.g. collagen type IV (CoIV) and elastin. Other resident proteins of the ECM are collagen type V, and entactin (Foidart et al., 1980; Bender et al., 1981; Kanwar & Farquhar, 1979). The underlying reticular layer, if present, contains interstitial collagen types I and III produced by both ECs and smooth muscle cells (Madri et al., 1980).

Heparan sulphate proteoglycans, the most abundant of the GAGs, compose much of the ECM and BM of endothelial cells and vascular smooth muscle cells (Fransson, 1987). GAGs and other proteoglycan molecules form a highly hydrated gel-like ground substance in which fibrous proteins are embedded. In the BM, heparan sulfate acts as an richly abundant glue by attaching to specific binding sites on collagen, laminin, and fibronectin (Gallagher et al., 1986; Saunders & Berndield, 1988) (fig. 6, page 18) with especially strong ties to LN and CoIV (75). Among the five collagen molecules, collagen type IV is strictly within the basement membrane where it serves to maintain structural integrity of the vessel wall. It is thought to form the core of all basal laminae. In a vessel wall of normal architecture, FN and LN are found in the subendothelium acting as cellular adhesion proteins. Using specific binding arms, LN attaches the EC primarily to the plasma membrane side of the BL as well as binding to collagen (preferentially

CoIV)(75); FN uses receptors called integrins to help bind matrix molecules and connective tissue on the other side. FN can bind to collagen type I or V and is also widely distributed in connective tissue. These rich attachments must be dissolved by proteolytic enzymes at the start of the angiogenic process before ECs may migrate. In respone to angiogenic stimuli, endothelial cells first secrete plasminogen activator and a variety of proteolytic enzymes causing fragmentation of the BM (Folkman, 1982).

Systematic studies on endothelial cell cultures have been instrumental in illustrating the function of many of the components of the ECM. When cultured on plastic, cells normally are contact-inhibited and show cobblestoning morphology. However, on a fibronectin, interstitial collagen type I or collagen type III substrate, these cells have increased proliferation over differentiation (83-85, 27, 77). In further contrast, if cultured on laminin or collagen type IV, the cells attach, differentiate, form capillary tube-like structures (83)(Kubota et al., '88), and their proliferation is suppressed. When antiserum is used to two different regions of the laminin molecule, experiments show that blocking one region, the RGD-integrin sequence, will inhibit cell-substrate adhesion and the cells will float in the medium, while blocking of another region, the YIGSR sequence, will inhibit cell-cell interaction and tube-formation is lost (77, 79). It appears that LN and CoIV are associated with differentiated mature (or maturing) vessels and FN and collagens I and III are important for proliferating or new vessels.

Light and EM studies showed that migration of ECs is an early event of angiogenesis (Cliff, 1963; Folkman, 1984). Both in situ fusion of hemangioblasts and invasion of sprouting capillaries require EC migration. Similar to other important cell migrations, e.g., neural crest cell migration (75), EC migration and differentiation are in part controlled by cell substrate adhesion molecules. Risau & Lemmon (1987) investigated ECM-EC interactions in early vasculogenesis and angiogenesis in the chick embryo. Using antibody labelling of FN and LN, they found that blood islands expressed high levels of FN but not LN, as did the intraembryonic vessels of the 6d old chick embryo. Laminin expression started on day 8 and by day 10, an "adult-like" distribution was found in the vascular wall (22). Simultaneously, angiogenesis was studied in the developing brain and found to display a similar progression of fibronectin to laminin expression as vessels matured. They conclude that "immature capillaries migrate and proliferate in a FN rich ECM, which is subsequently remodeled acquiring BM-like characteristics...laminin expression is an

early indication of vascular maturation." (22). Through in vitro cell culture studies described above and similar in vivo, other researchers echo this theory (77, 86-88).

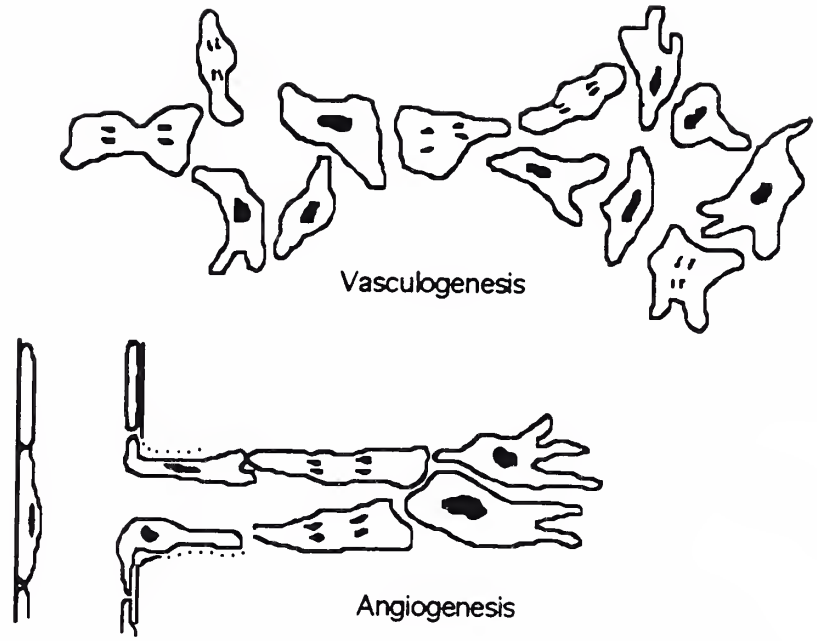


Figure 5. Vasculogenesis is the in situ differentiation of endothelial cells to form vessels. Angiogenesis is the sprouting of new capillaries from preexisting vessels. (Schematic diagram by JYL)

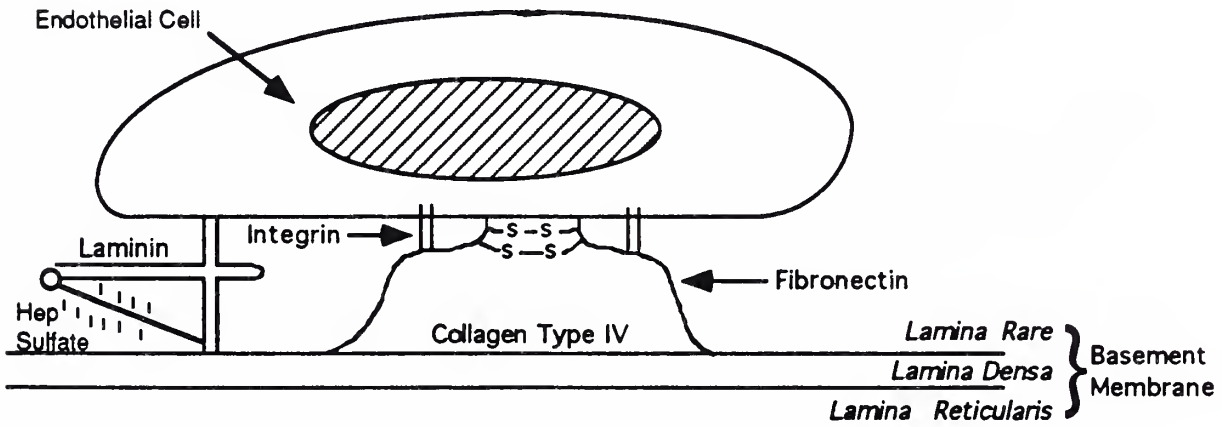


Figure 6. The basement membrane is the specialized portion of the extracellular matrix that lies on the basal aspect of endothelial and epithelial cells. It is composed of many different molecular species, mainly adhesion proteins, structural proteins, and glycosaminoglycans. (Schematic diagram by JYL)

D. Implications for Vascular Disorders in the Central Nervous System

The impact of angiogenesis research lead to the search for angiogenesis factors in a plethora of other diseases that are vascular disorders by nature. In the infantile and childhood hemangiomas, for example, which are vascular dysplasias that undergo discrete phases of growth and involution, there appeared to be a high expression of both bFGF and VEGF during the proliferating phase that was not seen in the involuting and involuted phases (89). In another vascular disease called Hemorrhagic Hereditary Telengiectasia (HHT) type 1 (also known as Osler-Weber-Rendu disease), patients have recurrent hemorrhages from multisystemic vascular dysplasias that include pulmonary arteriovenous malformations (PAVMs), mucosal, dermal, and visceral telengiectasias, cerebral AVMs and aneurysms. Studies indicate that the earliest pathological event is the dilation of post-capillary venules. Recently, the defective gene in this autosomal dominant disease was identified to be endoglin, which is a cell-surface binding protein for TGF-b. This growth factor, among other functions, is a potent angiogenic factor and controls ECM production from endothelial cells, smooth muscle cells, and pericytes (90). Cells deficient in this receptor may form altered cell adhesion properties and grow into abnormal vessels such as seen in HHT.

With such abundant literature about angiogenesis and angiogenic factors, endothelial cell migration and interaction with ECM, and discovery of angiogenic factors in various vascular disorders, one may quickly wonder if the vasculature of the brain is similar enough to that of non-nervous system tissue to be able to extrapolate research from other parts of the body. Indeed, it is, and the extrapolation has already begun. Brain vasculature is similar to that in other tissue (91, 75, 87) even at the scanning electron microscope level (76). In the normal adult nervous system, the cerebral vascular BM and the glial limitans externa have been well characterized and, like other tissues, found to contain collagen types I, III, and IV, FN, LN, and heparan sulfate protetoglycan (75, 87, 91-92)(Petou et al., 1980). Cell culture studies have determined that Schwann cells, immature astrocytes, and leptomeningeal cells can synthesize basement membrane molecules such as CoIV, LN, CoV, entactin, and heparan sulfate in vitro (93-97).

Recent investigators have begun the search for angiogenic growth factors and extracellular matrix disruptions in vascular disorders that occur in the CNS. An idiopathic disease called moyamoya (98) is characterized by gradual stenosis of the intracranial arterial trunk. This stenosis is thought to result in

extensive compensatory collateral vascular proliferation, including the fine network of vessels at the base of the brain, which show up on angiogram as a "puff of smoke." Intense bFGF staining was found in the ECs and VSMCs of the superficial temporal arteries and dura mater samples from four patients. In contrast, control tissue showed only faint and scattered immunoreactivity. The authors speculate that increased bFGF expression is responsible for the intimal thickening as well as the enhanced angiogenesis that is seen in this disease. In support of this theory, Lindner & Reidy (1991) held clinical trials that suggest bFGF's role in arterial restenosis after balloon angioplasty and arterectomies. By systemically injecting anti-bFGF neutralizing antibodies prior to the procedure, they demonstrated marked inhibition of medial vascular smooth muscle proliferation at the site.

E. Hypotheses & Objectives

AVMs and CMs of the central nervous system have been recognized for decades for their distinct clinical behavior and well-characterized features. However, the investigation into mechanisms of their pathogenesis and subsequent evolution has lagged far behind. Though both VEGF and bFGF are expressed abundantly during embryogenesis (29-30), these growth factors are not abundantly expressed in normal adult cerebral vasculature (40, 98, 99). Recent work by other investigators presents evidence suggesting that these angiogenic growth factors may play a role in the pathogenesis of moyamoya, childhood hemangioma, and atherosclerosis. We speculate that these growth factors may also be expressed in AVMs and CMs of the central nervous system.

In this medical degree thesis, we propose a novel strategy of analyzing the role that VEGF and bFGF may play in the functional biology of CNS vascular malformations, a possibility which has never before been explored. Using two-stage immunohistochemical technique, we subject twelve surgically resected specimens to a selected battery of antibody stains. We explore the presence of angiogenic factors using antibodies to VEGF and basic FGF. In addition, we also examine the structural biology of these lesions using immunostaining for selected proteins: laminin, fibronectin, collagen type IV, and alpha smooth muscle actin. Laminin and collagen type IV are associated with more mature vessel structures, whereas fibronectin is associated with proliferating, less mature vessel walls. Alpha smooth muscle actin is mainly a marker for the smooth muscle layers of vessel walls and collagen type IV is strictly localized to mature

basement membranes. These proteins, unlike enzyme systems, are stable during the period between resection and tissue fixation and therefore subject to detection by the immunohistochemical technique.

Angiogenic growth factors, if found to be expressed in AVMs and CMs, may interact in distinct ways with the surrounding extracellular matrix proteins to produce the vascular abnormalities that characterize these lesions. Previous reports from our laboratory suggest that CMs lack mature vessel wall elements and contain a fibronectin-rich extracellular matrix as compared to AVMs. Angiogenic factors in the context of these factors may produce proliferating vessels that lack wall integrity, thereby predisposing them to some of the clinical features seen in CMs. The growth factors in AVMs, however, may produce vessels that are more "mature", and different in histology from CMs, but predisposed to pathology of their own primarily because of the high-flow dynamics characteristic of these lesions. This strategy of molecular dissection using immunohistochemistry for angiogenic growth factors and matrix proteins attempts to identify unique features associated with and possibly mediating the biologic behavior of individual lesion types.

II. MATERIALS & METHODS

Twelve cases of central nervous system vascular malformations underwent resection for conventional indications as summarized in Table 1 (page 37). Five were identified as cavernous malformations according to histologic criteria (1, 2), all without recent gross hemorrhage. Seven cases were characterized as AVMs by histologic and radiographic criteria (1, 2), of which one was angiographically occult (small "cryptic" AVM). One AVM (case 2) was associated with systemic manifestations of hereditary hemorrhagic telengiectasia (Osler-Weber- Rendu) disease in the same host. In one case (Case 7), only the arterialized draining vein of the spinal dural AVM was available for this study. Three cases underwent preparatory embolization at 3, 8, and 11 days, respectively, prior to resection.

All surgical specimens were formalin-fixed and paraffin-embedded into tissue blocks. For each case existed one tissue block, of which multiple sequential sections of 6 micron thickness were cut and mounted onto glass slides. These slides were then deparaffinized in xylene, and rehydrated in preparation for immunohistochemical studies for vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), laminin (LN), fibronectin (FN), collagen type IV (CoIV), and alpha smooth muscle actin (ASMA). Using the tested protocol outlined below, one slide specimen per case was immunostained for each target antigen examined.

Immunostaining for Angiogenic Growth Factors (VEGF, bFGF)

After deparaffinization, the tissue sections were incubated in 3% hydrogen peroxide in methanol for 30 minutes to quench endogenous peroxidase activity and thereby minimize nonspecific background staining. All incubation steps are performed in a humidity chamber. A 10 minute rinse with Tris phosphate buffered saline (TBS, pH=8.2), and a 20 minute blocking step with normal goat serum (NGS) followed, both on a gentle shaker at room temperature. The primary polyclonal IgG antibody (provided by Dr. Daniel T. Connolly, Ph.D., Monsanto Company, St. Louis, MO) (53) was raised in rabbits against the 21-amino acid NH2-terminal sequence of guinea pig VPF/VEGF. Both the primary and the preimmune rabbit IgG (control primary) were prepared at 1:800 dilution in a phosphate buffered solution (PBS, pH=7.5) with 1% bovine serum albumin (BSA) and 0.1% Tween detergent. These were applied for 20 hours at 4 degrees Celsius. The slides were then rinsed again with TBS for 10 minutes. The next three steps are part of the

avidin-biotin-enzyme complex (ABC) method developed by Hsu et al. (100), using the Vectastain Elite Rb IgG ABC kit and DAB (3,3-diaminobenzidine tetrahydrochloride) Peroxidase Substrate Kit (Vector Labs, Burlingame, CA) according to kit instructions. The sections were incubated with the anti-Rb IgG biotinylated secondary antibody (1:200 dilution) for 30 minutes, rinsed with TBS for 10 minutes, then incubated with the ABC complex solution for 30 minutes, all on a gentle shaker at room temperature. The DAB peroxidase substrate solution was mixed according to instructions and applied to sections for 8 minutes. The enzymatic reaction was stopped with distilled water, and the sections rinsed with a 5 minute water bath. The sections were then counterstained using hematoxylin only (for better cytoplasmic visualization), dehydrated, cleared in xylene, mounted under coverslips, and examined microscopically. All specimens were run in parallel and compared with negative controls using preimmune rabbit serum as primary antibody.

The immunostaining technique for bFGF was similar to that for VEGF with the following exceptions. The primary, a monoclonal (clone 3H3) IgG antibody, was affinity-purified from Balb/c mice immunized with recombinant human bFGF (Oncogene Science, Cambridge, MA)(Watanabe et al., 1991) and used at a 1:50 dilution. Normal horse serum was used for the blocking step, preimmune mouse IgG served as the control primary (at a matching dilution), the Vectastain Elite Mouse IgG ABC kit contained the appropriate anti-Ms IgG biotinylated secondary antibody solution, and the DAB reaction took 7 minutes.

Immunostaining for Extracellular Matrix Proteins (LN, FN, CoIV, ASMA)

After deparaffinization and rehydration, the sections for laminin, fibronectin, and collagen type IV underwent enzyme pretreatment to "unmask" protein antigenicity (103), a routine step to enhance certain stainings when using paraffin embedded tissues. Laminin studies required a 7 minute incubation with 0.4% protease/$CaCl^2$/TBS solution. Fibronectin and CoIV required a 7 minute treatment with 1.0% trypsin/$CaCl^2$/TBS. (Both enzymes from Sigma, St. Louis, MO.) ASMA studies required no enzyme digestion. Enzymatic steps were stopped with TBS and sections then rinsed under running water for 7 minutes. All sections were then covered with normal goat serum for 20 minutes at room temperature to block nonspecific staining prior to the primary antibody incubation step.

The primary antibody sources and target tissues were as follows. A monoclonal antibody (clone 4c7, subclass IgG2alpha) against human laminin was raised in mice to react with the terminal globular domain of the A chain of human laminin and block the neurite stimulating activity of the glycoprotein (Dako, Carpinteria, CA). LN-Ab is expected to stain basement membranes of endothelium and epithelium regardless of antibody concentration. It could stain cytoplasm of cells faintly in formalin fixed tissues because LN is produced by these cells. A polyclonal antibody against fibronectin isolated from normal human plasma was purified from rabbit antiserum (Dako). FN-Ab normally stains vessels and connective tissue. A monoclonal antibody (isotype IgG1, kappa) against purified pepsin fragments of human type IV collagen from human kidneys was raised in mice and recognizes conformational epitope on a helical part of native collagen IV (Dako). CoIV-Ab stains only cellular basement membranes. A monoclonal antibody (clone 1A4, isotype IgG2a, kappa) against N-terminal decapeptide of alpha-smooth muscle actin was raised in mice (Dako). ASMA-Ab stains smooth muscle cells (SMC) of vessels and different parenchymes without exception; intensity may vary according to the amount of ASMA present in the VSMC, myoepithelial cells, pericytes, sometimes myofibroblasts in benign and reactive fibroblastic lesions. It does not cross-react with actin from fibroblasts, striated muscle, or myocardium.

The primary antibody solutions and incubation conditions were as follows. The anti- laminin antibody was diluted 1:200 in 1% BSA/0.1% Tween detergent/PBS solution. Sections were incubated for 20 hours at 4 degrees Celsius. The anti-fibronectin was diluted 1:2000 in BSA/Tween/PBS solution and sections were incubated for 1 hour at 37 degrees Celsius. The anti-collagen IV was diluted 1:25 in BSA/Tween/PBS solution and sections were incubated for 1 hour at 37 degrees Celsius. The anti-ASMA was used at 1:50 dilution and sections incubated for 30 minutes at room temperature. All incubation steps were done in a humidity chamber.

Next, the sections were rinsed for 10 minutes with TBS on a gentle shaker. The following steps were performed using the Kirkegaard Monoclonal ABC Kit for laminin, CoIV, ASMA; Kirkegaard Polyclonal ABC Kit for fibronectin (Kirkegaard and Perry Laboratories, Inc; Gaithersburg, MD); and Vector Alkaline Phosphatase Substrate Kit (Vector Labs, Burlingame, CA). The goat anti-mouse IgG biotinylated secondary antibody solution was applied for 30 minutes (goat anti-rabbit secondary for FN) at room

temperature, followed by a 10 minute TBS rinse and a 30 minute incubation with the streptavidin-phosphatase solution (ABC complex) and another 10 minute TBS rinse. Finally, the alkaline phosphatase substrate solution was added and the reaction allowed to proceed for 8 minutes for LN and CoIV, and 2 minutes for FN and ASMA, before being stopped with distilled water. Each specimen was run in parallel with monoclonal and polyclonal negative primary antibody controls (Dako). All sections were then counterstained with hematoxylin only, dehydrated, cleared in xylene, and sealed under coverslips.

The results of immunostaining were evaluated in each specimen under the microscope. Staining was reported as nil (0), faint (1+), or intense (2+). The pattern and distribution of espression of the various proteins were documented for each lesion and contrasted among lesion types. The pattern of staining for each experiment will be described according to location within the lesion architecture. The vessel layers referred to throughout this thesis are: endothelial layer lining the vascular lumen, the subendothelial layer (just below the previous layer, the interface between endothelium and media), the vascular media or adventitia of AVM vessels (recognized through their fairly typical anatomy), the intercavernous matrix of CMs (since CMs do not exhibit recognizable media or adventitia), and the perivascular tissue (brain parenchyma within and surrounding the lesion).

The original protocol for all immunohistochemical experiments in this study was developed by the student author. This protocol was also used in another study from our laboratory (99). All reagents were available from commercial vendors as noted above. All immunostaining experiments, photographs of results, and figures used in this thesis were done by the student author. Data analysis of the immunohistochemical staining results was done jointly by the authors and Drs. Kim and Harrington of Neuropathology. The work in this thesis is currently in press for May, 1996.

III. RESULTS

Angiogenic Growth Factors

A summary of the angiogenic factors experiments is presented in Table 2 (page 38). All twelve lesions stained positively for VEGF with the AVMs staining somewhat more intensely and diffusely. In both types of lesions, the staining was patchy in distribution and demonstrated predominantly in the endothelium and perivascular tissue (fig. 7, 8 on pages 28-9). Among the AVMs, there was no differential expression between arteries and veins, with the exception of one lesion that expressed VEGF predominantly among its venous components. Within individual CMs, small and large caverns had an equal propensity for VEGF expression.

Of the 7 AVMs examined, 4 stained positively for bFGF. The staining was seen primarily in the media of larger vessel walls (fig. 9, page 30) with only one lesion exhibiting expression by endothelial cells. The angiographically occult AVM did not express bFGF. Four of the 5 CMs demonstrated faint expression of bFGF within the lesion, and one additional CM was negative within the lesion but positive in scattered swollen axons surrounding the lesion. Staining was patchy, involving some caverns but not others and was noted both within the endothelial layer and in the perivascular tissue. There was no appreciable difference in VEGF or bFGF expression among lesions which had recently hemorrhaged or among embolized lesions. A specimen that consisted entirely of an arterialized leptomeningeal vein from a dural AVM expressed both angiogenic factors. VEGF staining was diffuse but appeared most intense near the endothelium, while bFGF expression was limited to the cytoplasm of scattered fibroblasts and myocytes in the media (fig. 10, pages 31-2).

Extracellular Matrix Proteins

Results of the immunostaining for selected structural and matrix proteins are summarized in Table 3 (page 39). The AVMs exhibited positivity for laminin in vessels in 3 of 7 lesions studied (fig. 11, page 33). By contrast, the vessels of the CMs did not demonstrate laminin expression; although relatively normal vessels in the parenchyma surrounding some of the lesions stained positively (not shown). Among the AVMs that exhibited laminin expression, the staining was patchy and involved primarily the thinner walled

larger vessels, and more uniformly small vessels in and around the lesion. The specimen of the arterialized vein did not reveal laminin immunoreactivity. Conversely, although none of the CMs demonstrated laminin expression, there was intense and diffuse fibronectin expression throughout the CMs (fig. 12, page 34). All AVMs studied exhibited faint and patchy staining for fibronectin (not shown).

Collagen type IV expression was noted to be intense thoughout the 12 vascular malformations studied. Staining was limited predominantly to the subendothelium within the CMs (fig. 13b, page 35). In contrast, AVMs demonstrated positivity throughout the media as well as the subendothelium (fig. 13a, page 35). Alpha smooth muscle actin had the same pattern and degree of staining in the AVMs (fig. 14a, page 36). Within CMs, ASMA was noted to be diffuse and intense. However, staining was mainly within the endothelial and subendothelial layers. In 2 of the CMs, ASMA expression was mainly in the smaller cavern walls and absent in the larger caverns (fig. 14b, page 36).

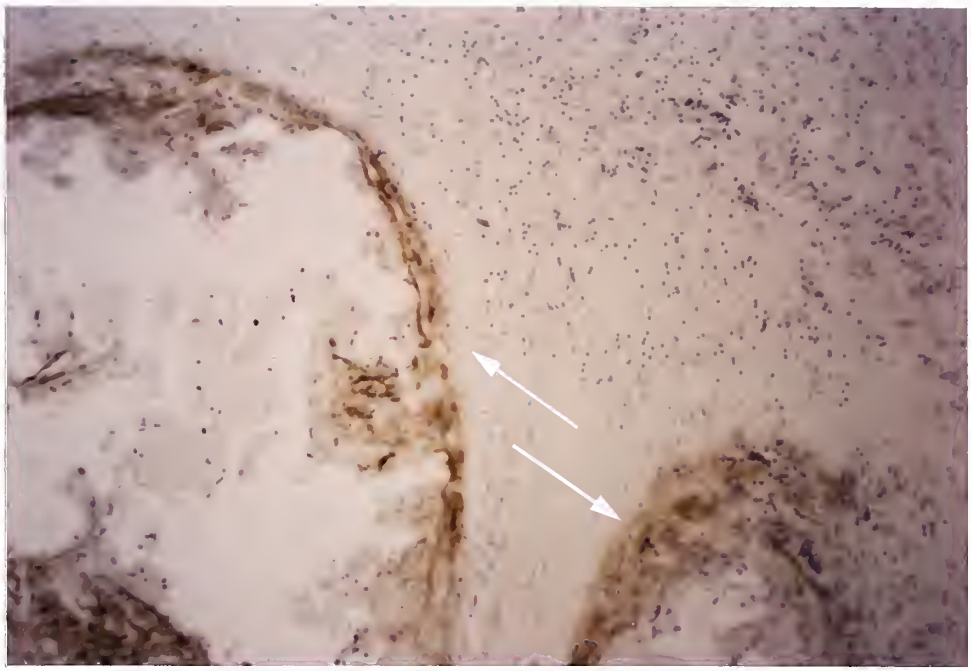
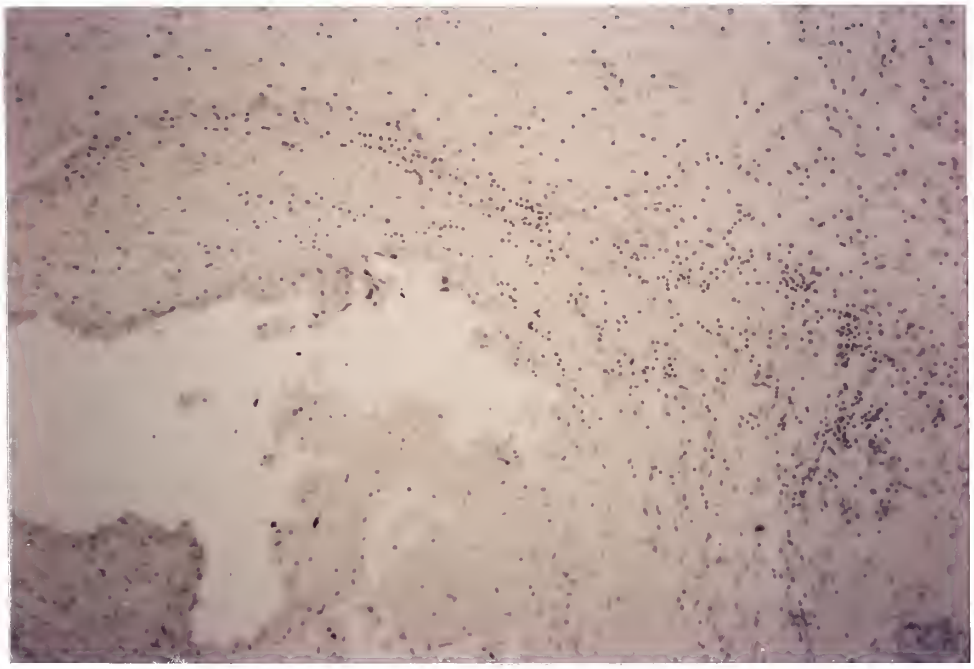


Figure 7. AVM a) VEGF staining was mainly in the endothelium and perivascular tissue. (10X)



b) Control (10X)

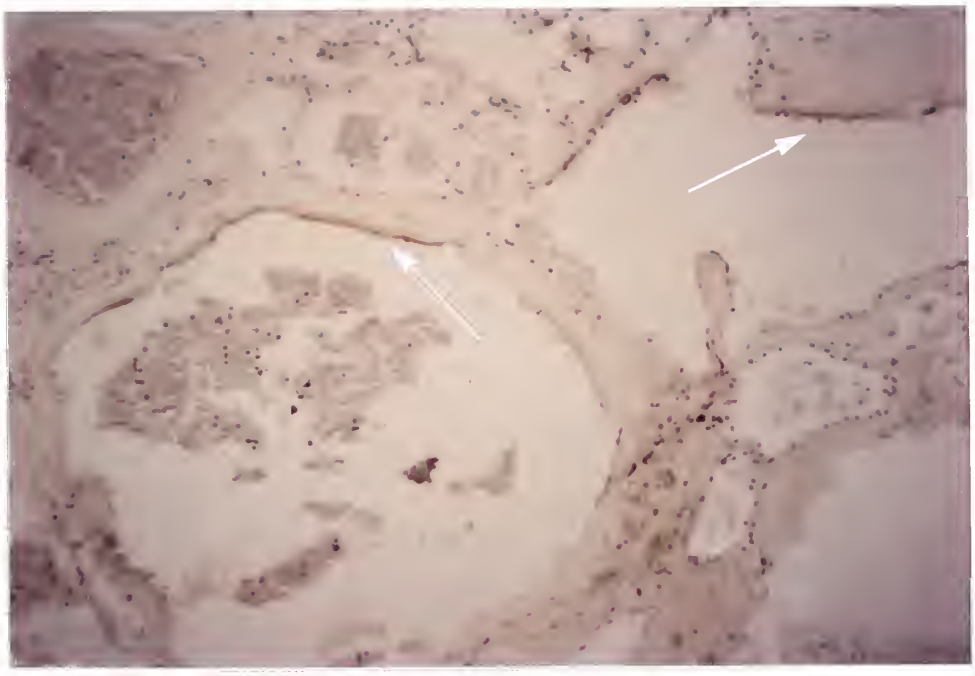
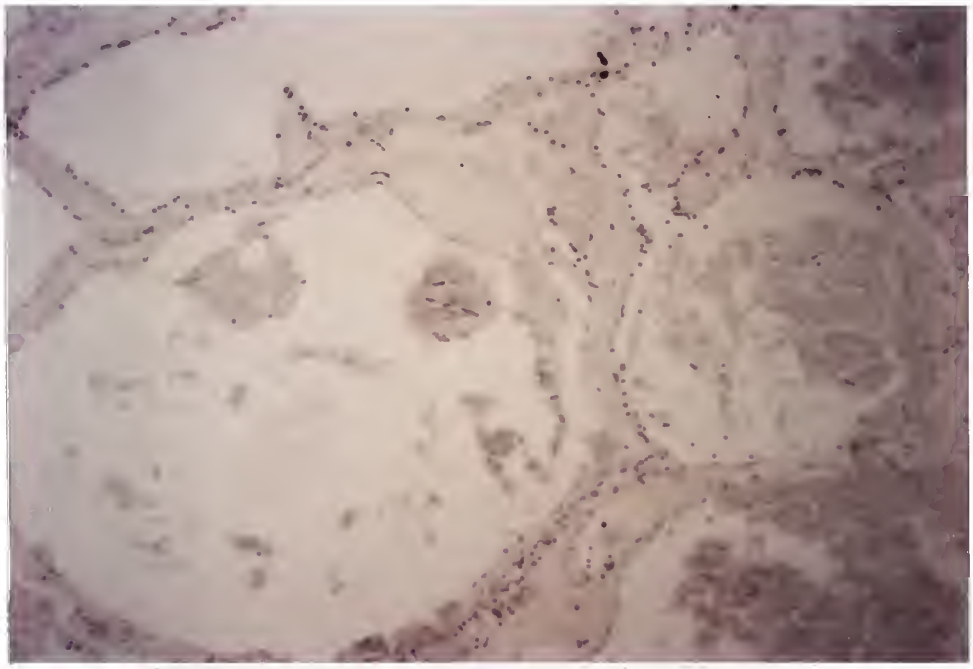


Figure 8. CM a) VEGF staining in the endothelium and intercavernous matrix. (10X)



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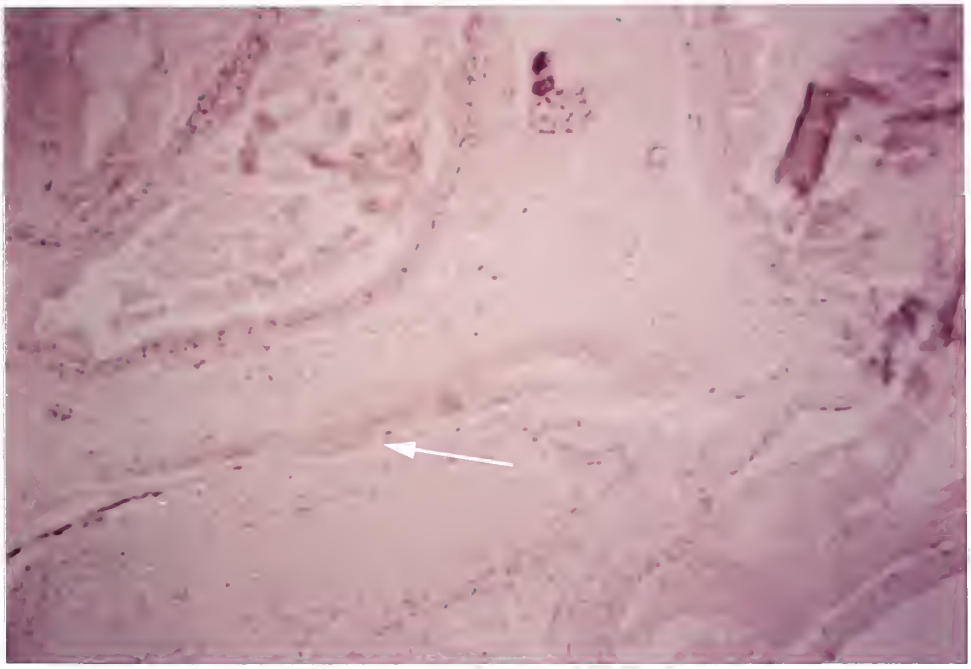
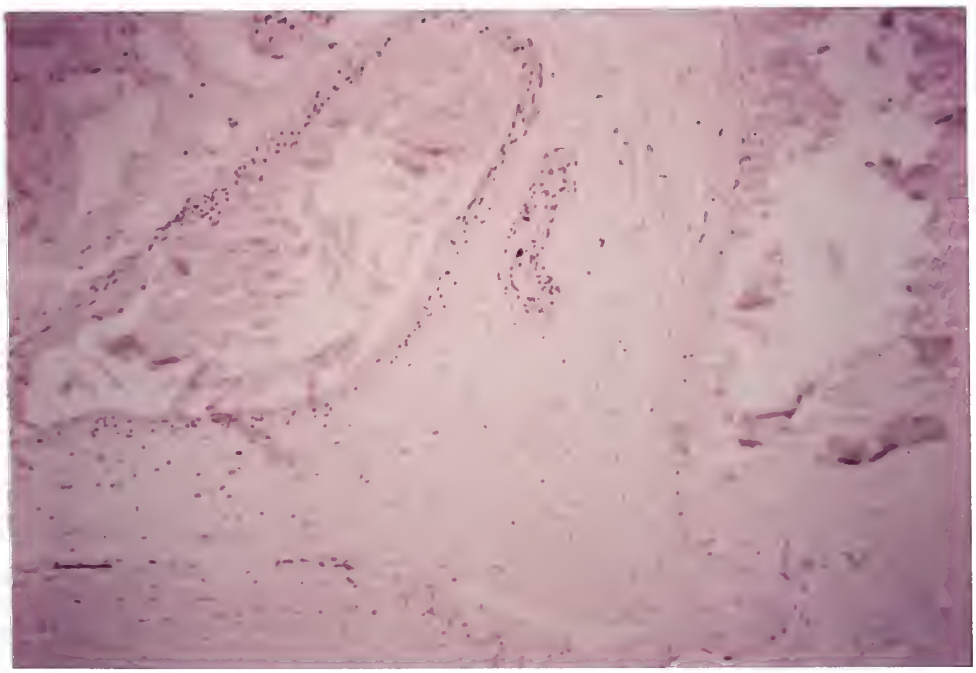


Figure 9. AVM a) Basic FGF was faintly positive mainly in the media. (10X)



b) Control (10X)

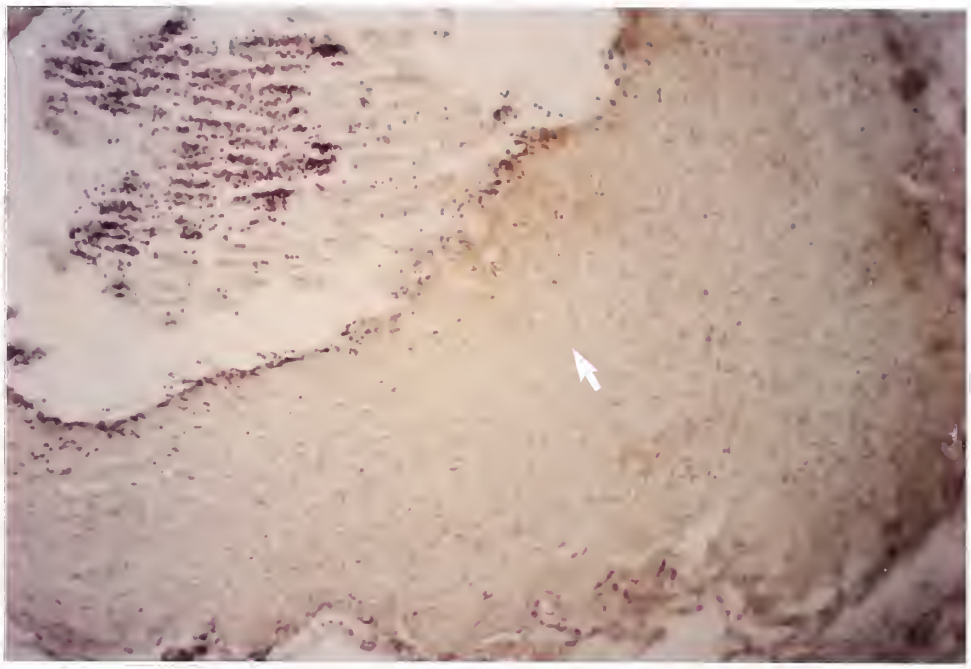
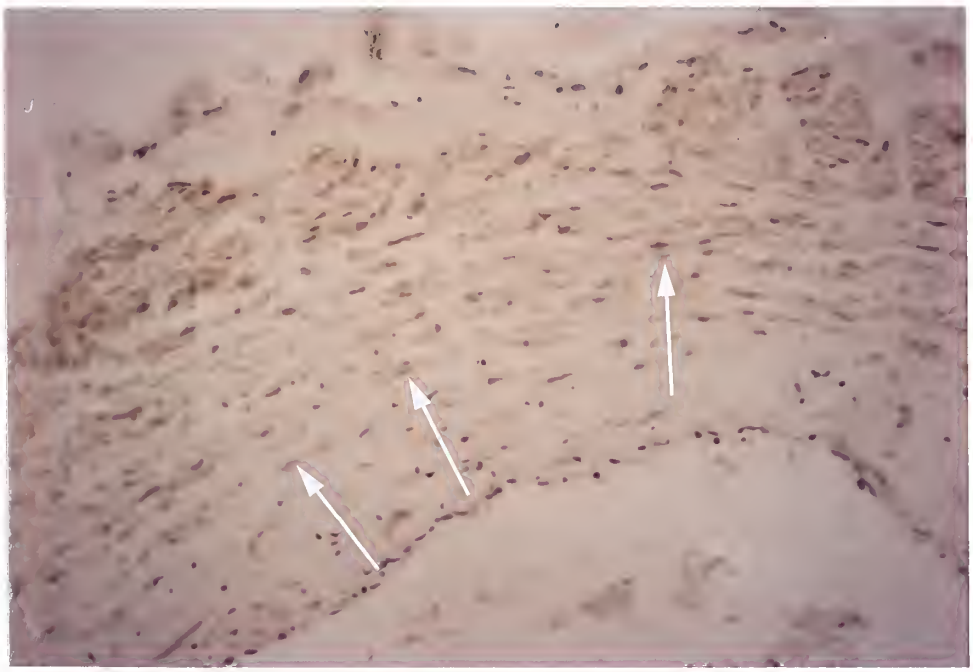
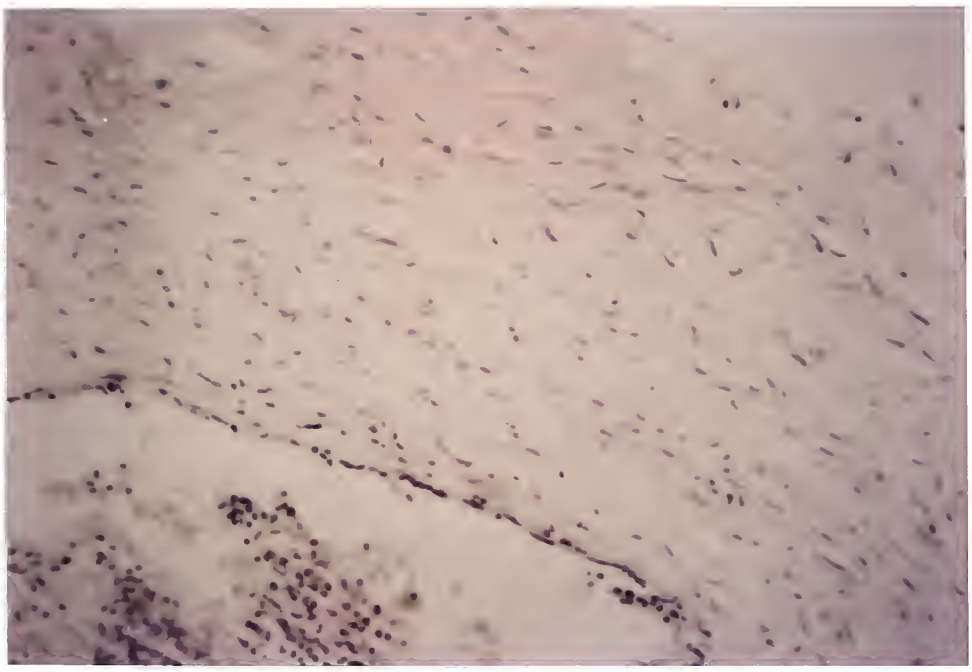


Figure 10. Draining vein of dural AVM a) VEGF staining is diffusely positive, more intense near the endothelium. (10X)



b) Basic FGF expression is faint, in the cytoplasm of scattered fibroblasts and myocytes in the media. (20X)



c) Control (20X)

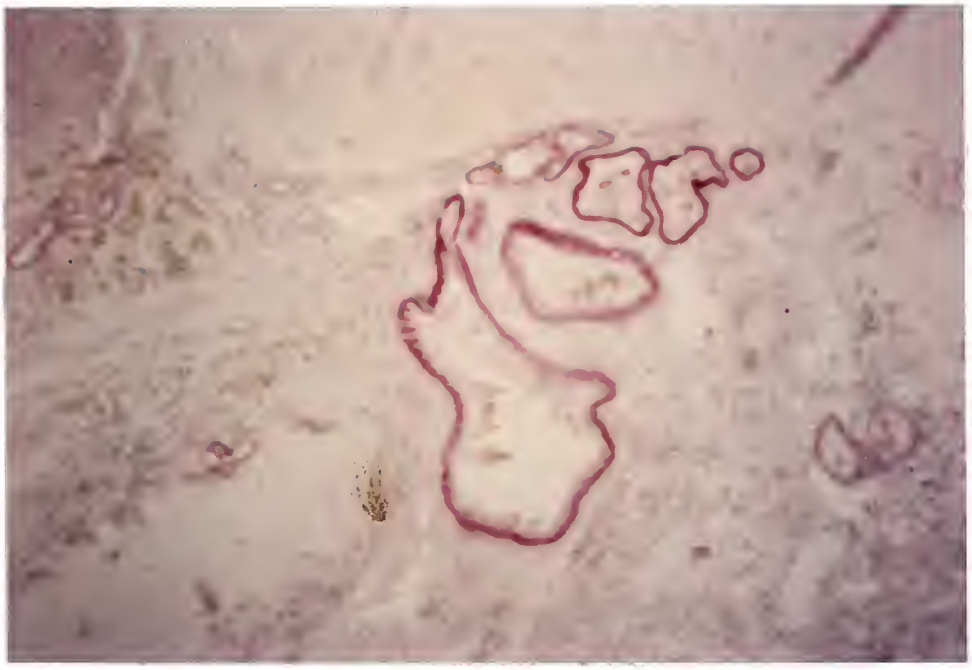
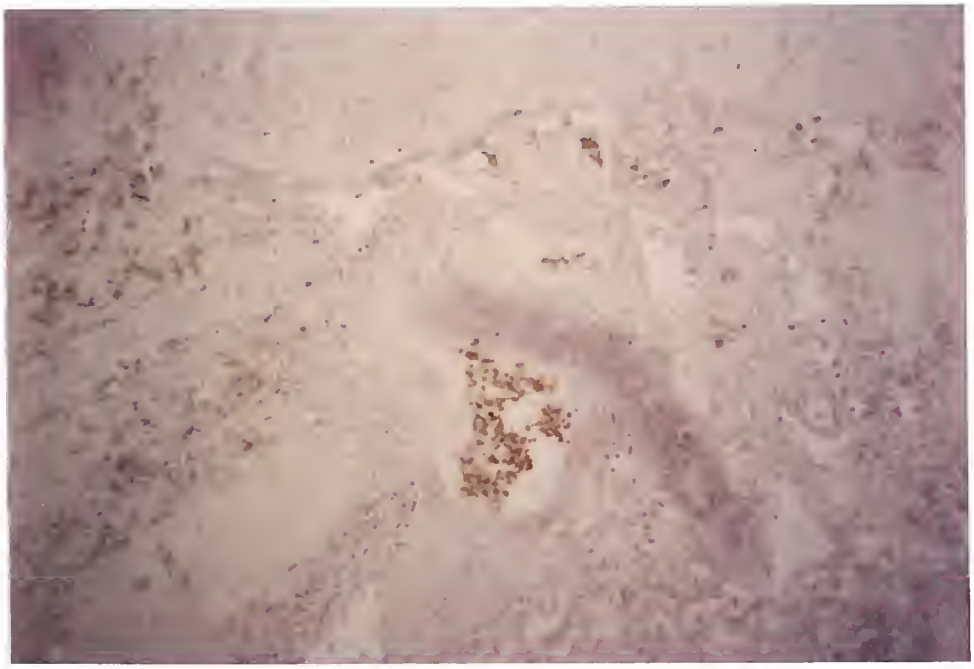


Figure 11 AVM a) LN staining in vessels of varying sizes. (10X)



b) Control (10X)

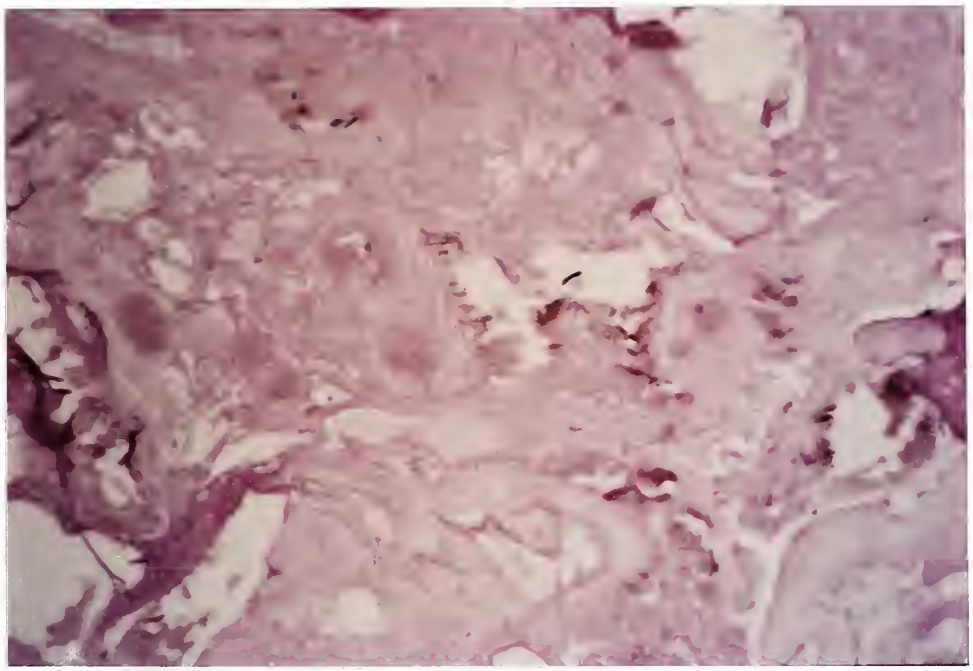
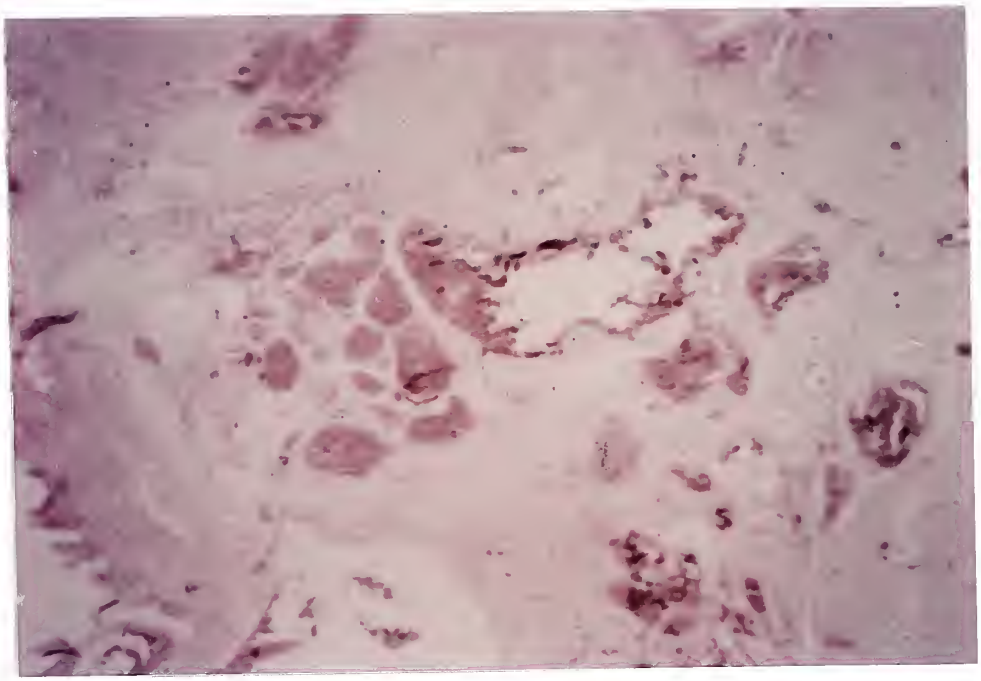


Figure 12. CM a) FN staining is diffuse and intense throughout the lesion. (4X)



b) Control (4X)

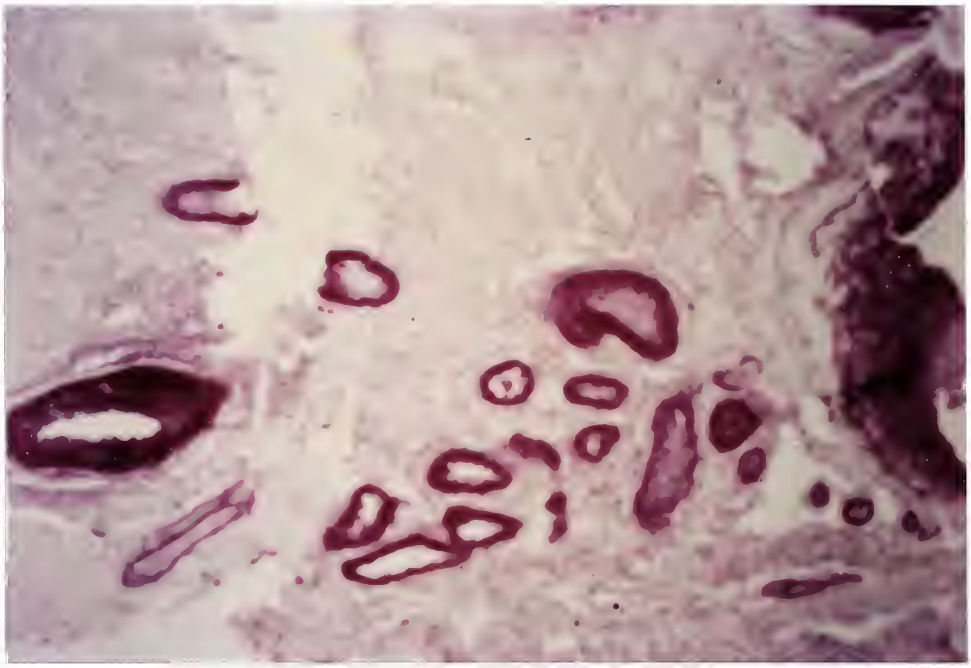
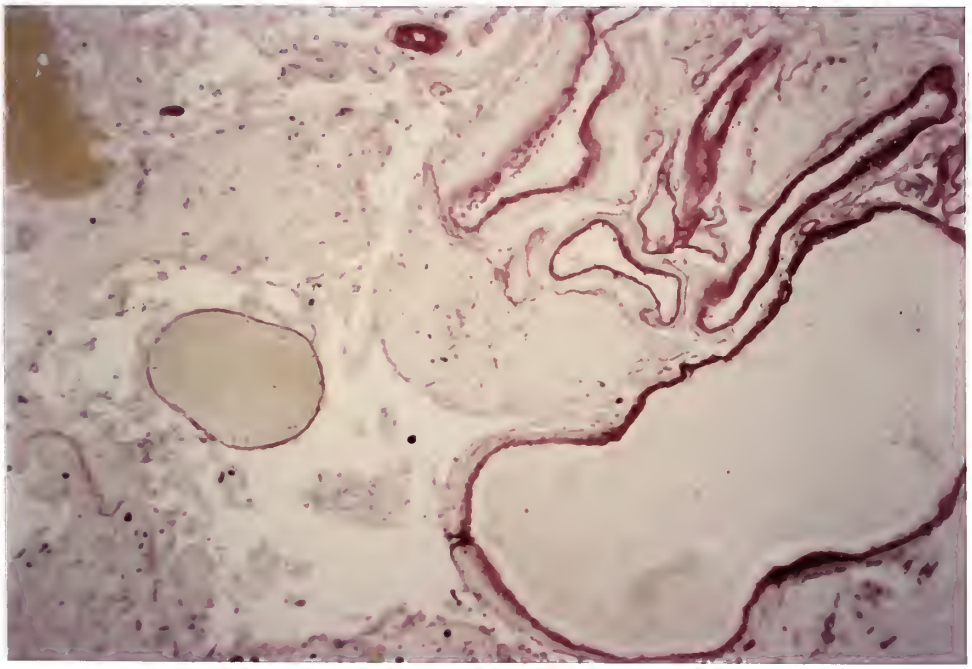


Figure 13. a) AVMs showed CoIV staining in the subendothelium and throughout the media. (10X)



b) CMs expressed CoIV predominately in the subendothelium. (10X)

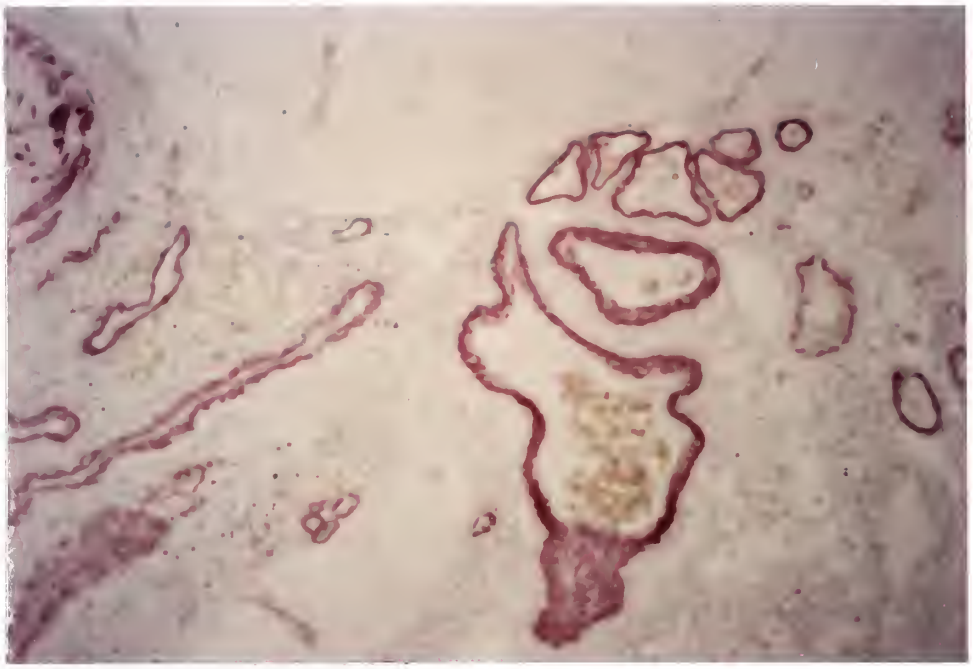
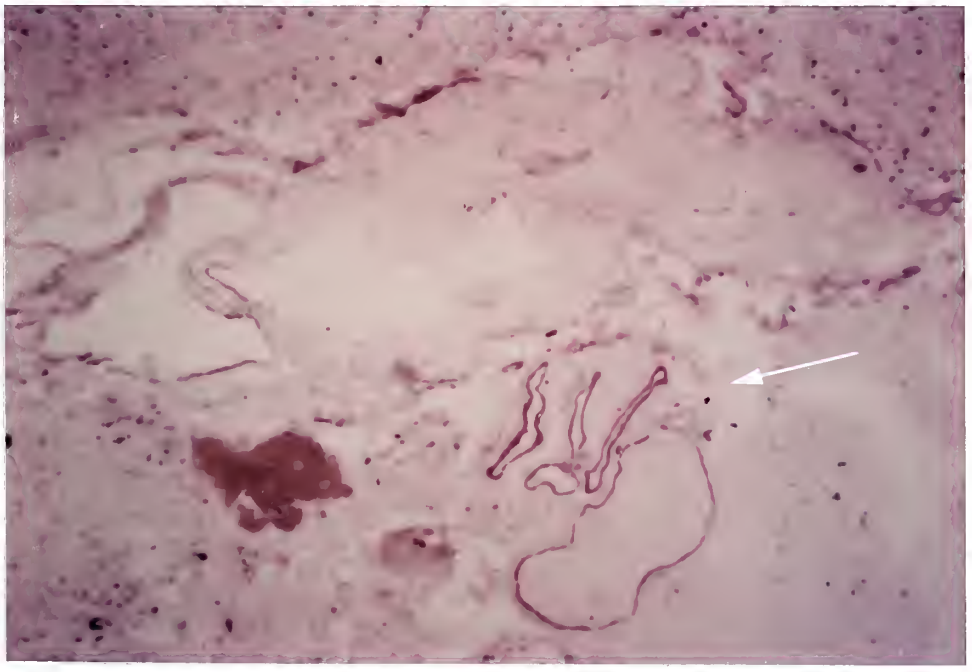


Figure 14. a) AVMs exhibited ASMA expression in the subendothelium and throughout the media. (10X)



b) CMs demonstrated ASMA mainly in the endothelial and subendothelial layer. In this lesion, notice the positivity in smaller but not larger caverns. (10X)

TABLE 1
CLINICAL AND LESION FEATURES OF 12 EXCISED CNS VASCULAR MALFORMATIONS

Case #/ Age/ Gender	Prior hemorrhage (interval til excision)	Angiography	Indications for Excision	Prior Embolization	Lesion Histology
1. 43/ M	No	AVM	Hemorrhage from associated arterial aneurysm	Yes	AVM
2. 52/ M	No	AVM	Intractable epilepsy	No	AVM
3. 38/ F	No	AVM	Headaches	Yes	AVM
4. 31/ M	Yes (10wks)	AVM	Hemorrhage; epilepsy	Yes	AVM
5. 41/ F	No	AVM	Headache	No	AVM
6. 18/ M	Yes (9mos, 2wks)	Occult	Hemorrhage	No	AVM
7. 47/ M	No	Dural AVM	Paraparesis	No	Arterialized vein
8. 26/ F	Yes (6mos)	Occult	Pontine syndrome	No	CM
9. 42/ F	Yes (12yrs)	Occult	Epilepsy	No	CM
10. 18/ F	No	Occult	Epilepsy	No	CM
11. 53/ M	No	Occult	Epilepsy	No	CM
12. 48/ F	No	Occult	Epilepsy	No	CM

TABLE 2
IMMUNOSTAINING FOR ANGIOGENIC GROWTH FACTORS IN 12 CNS VASCULAR MALFORMATIONS

Case #/ Lesion	VEGF Intensity*	VEGF Distribution	bFGF Intensity*	bFGF Distribution
1. AVM	2+	Perivascular matrix, subendothelium, media gliotic white matter	2+	Perivascular matrix, endothelium, media
2. AVM	2+	Perivascular matrix, endothelium, subendothelium, media, perdominately in veins	2+	Perivascular matrix, endothelium, media
3. AVM	2+	Perivascular matrix, endothelium	1+	Media
4. AVM	2+	Perivascular matrix, endothelium, subendothelium, media, gliotic white matter	0	
5. AVM	1+	Perivascular matrix, gliotic white matter	0	
6. AVM	2+	Perivascular matrix, endothelium, subendothelium	0	
7. AVM	2+	Endothelium, subendothelium, media	1+	Fibroblasts
8. CM	1+	Intercavernous matrix, endothelium of small caverns, small perilesional capillaries	1+	Intercavernous matrix
9. CM	1+	Intercavernous matrix, endothelium, perilesional white and gray matter	0	
10. CM	2+	Intercavernous matrix, endothelium; subendothelium, small and large caverns	1+	Endothelium, subendothelium
11. CM	2+	Intercavernous matrix, endothelium, subendothelium, perdominately large caverns, perilesional white matter	1+	Swollen perilesional axons, subendothelium
12. CM	1+	Perilesional white and gray matter	1+	Swollen perilesional axons, intercavernous matrix

*Grading of immunostaining intensity:
- 0 No perceptible increase from control
- 1+ Faint increase from control
- 2+ Marked increase from control

TABLE 3
IMMUNOSTAINING FOR STRUCTURAL PROTEINS IN 12 CNS VASCULAR MALFORMATIONS

Case #/ Type	Laminin Intensity*	Laminin Distribution	Fibronectin Intensity*	Fibronectin Distribution	Collagen IV Intensity*	Collagen IV Distribution	Smooth Muscle Actin Intensity*	Smooth Muscle Actin Distribution
1. AVM	1+	Small & large thin vessel walls	1+	Endothelium, subendothelium	2+	Endothelium, subendothelium, media	2+	Subendothelium, media
2. AVM	1+	Large thin vessel walls	1+	Endothelium, adventitia	2+	Endothelium, subendothelium, media	2+	Subendothelium, media
3. AVM	1+	Small & large thin vessel walls	1+	Endothelium, adventitia	2+	Subendothelium, media	2+	Subendothelium, media
4. AVM	0		1+	Endothelium	2+	Subendothelium, media	2+	Subendothelium, media
5. AVM	0		1+	Transmural	2+	Subendothelium, media	2+	Subendothelium, media
6. AVM	0		1+	Transmural	2+	Subendothelium, media	2+	Subendothelium, media
7. AVM	0		1+	Transmural	2+	Subendothelium, media	2+	Subendothelium, media
8. CM	0		2+	Transmural	2+	Subendothelium, intercavernous matrix	2+	Subendothelium, endothelium
9. CM	0		2+	Transmural, neg perilesional vessels	2+	Subendothelium, intercavernous matrix	2+	Endothelium, only thin walled caverns
10. CM	0		2+	Transmural	2+	Subendothelium, intercavernous matrix	2+	Endothelium, only thin walled caverns
11. CM	0		2+	Transmural, neg perilesional vessels	2+	Subendothelium, intercavernous matrix	2+	Endothelium, subendothelium
12. CM	0		2+	Transmural	2+	Subendothelium	2+	Endothelium, subendothelium

IV. DISCUSSION

The processes of new vessel formation by in situ differentiation of endothelial cells (vasculogenesis) during embryogenesis and the sprouting of new vessels from preexisting vasculature (angiogenesis) during organogenesis, wound healing, tumor growth, and inflammation have been studied extensively in the past two decades (21-5, 27-8, 87). It has become clear that angiogenesis occurs in discrete tightly controlled steps, including enzymatic breakdown of local basement membrane, migration of endothelial cells through altered matrix milieu, capillary tube formation, basement membrane synthesis, and subsequent maturation of neovessels. Diffusible growth factors mediating several of these steps such as endothelial cell proliferation, migration, adhesion, and permeability have been identified and isolated. Among these factors, bFGF has been shown to be a powerful mitogen of endothelial cells, fibroblasts, and myocytes and an effective inducer of angiogenesis in vitro and in vivo (27, 29-33, 35-7, 39-40, 46-8, 98). Basic FGF is also able to induce plasminogen activator, protease production, DNA synthesis, and migration of endothelial cells (Folkman, 1982)(31, 46-48).

VEGF, initially discovered as VPF, is another factor that promotes angiogenesis, and possesses the unique feature of being mitogenic strictly for endothelial cells (49-55, 62-3, 67-8, 73, 105). VEGF can directly promote premeability of vascular beds, through leakage at intercellular junctions, and is thought to play a key role in the initiation of angiogenesis, and in the abnormal vascular permeability associated with pathologic processes such as peritumoral brain edema (56-61, 64). VEGF is expressed during early phases of atherogenesis, and in various pathologic processes such as inflammation and tumor growth (27, 57-61). A variety of cells can synthesize and secrete VEGF under pathologic conditions, including endothelial cells, fibrocytes, myocytes, and astrocytes. VEGF receptors are exclusively expressed on endothelial cells throughout the normal tissues of the body (63). Recently, gene manipulation studies have illustrated the existence of at least four different VEGF receptors that are involved in discrete components of angiogenesis (69-71).

A. Angiogenic Growth Factors

VEGF and bFGF are expressed abundantly during embryonic development (29-30), but are absent in most normal adult vascular beds including cerebral vasculature (40, 99). In this study, we have

demonstrated VEGF expression in all twelve vascular malformations examined. VEGF was seen mainly in the perivascular tissue, subendothelium, and media of vessels of various sizes within AVMs. Positive staining was also present in the vascular adventitia and interstitium of perilesional gliotic brain. In the CMs, VEGF staining was seen in the intercavernous matrix and subendothelium of some caverns, but not all caverns within a given lesion. VEGF expression was also seen in the surrounding brain and around perilesional capillaries. This pattern of expression suggests diffuse upregulation of VEGF and possible involvement in an active angiogenic process.

Basic FGF was expressed in the majority of vascular lesions, but in a very different distribution than VEGF. In both lesion types, there was faint staining around individual myocytes and fibrocytes in the vascular matrix. In some cases, there was also some positivity in the endothelial layer. This is consistent with the bFGF's ability to act on a wider spectrum of target cells than VEGF, including ECs, fibrocytes, and myocytes.

The expression of VEGF and bFGF was not remarkably different in arterial compared to venous beds, nor was it limited to lesional vessels. Vascular smooth muscle cells (VSMC) are highly responsive to the mitogenic effects of growth factors (Cuevas et al., 1991) such as bFGF. Similar to the role that bFGF is thought to play in the thickening of vessel walls in response to hypertension (26-7) and idiopathic arterial stenosis in moyamoya, it is possible that bFGF may mediate the arterial vessel thickening. In response to the high pressure shunt in an AVM, the vein undergoes morphological changes that bFGF may also effect, producing the abnormal VSMC proliferation characteristics of the "arterialized vein" histopathology consistently seen in AVMs. Kittelberger et al. (1990) found that hemodynamic stress does induce extracellular matrix changes in the vessel wall of animal aneurysm models (106). The one arterialized vein specimen obtained from a dural AVM expressed both angiogenic factors. It is interesting to note that Jakeman et al. (1992) had found that the endocardium, more than elsewhere in the heart, showed the most VEGF-binding potential, suggesting that in a region of such high stress forces and erosion, VEGF may be involved in continual repair and maintenance of the endothelium (63).

Studies using a rat model of dural AVM by Herman et al. (1995) demonstrated that dural sinus hypertension, not sinus thrombosis alone, is important in creating dural AVMs (104). At the microscopic

level, Awad et al. (1990) theorize that when sinus occlusion limits normal venous drainage, microarteriovenous fistulas in the sinus wall open and develop into a dural malformation. Other investigators have described a dural AVM that appeared to induce cortical venous drainage (6) further illustrating the dynamic nature of these lesions. The finding of angiogenic factor expression in a dural AVM suggests that hemodynamic stress alone may induce upregulation of these factors in preexisting vessels, which may in turn mediate flow induced morphological changes seen in AVMs.

Positive staining for both VEGF and bFGF was also seen in vessels near but outside of the AVM nidus. These observations reflect the curious ability of AVMs to "recruit" neighboring ("en passage") vessels and functionally incorporate them into the lesion, thereby creating growth. Other investigators have reported cerebral AVMs that appear to acquire artery feeders from a different cerebral circulation over time (7). Studies using positron emission tomography (PET) have shown that brain tissue immediately surrounding the high-flow artery-venous shunt of an AVM is relatively ischemic, a phenomena called "steal" (5). Hypoxia, which is a stimulus for angiogenesis (77), has been shown to induce the upregulation of angiogenic factors (105), and therefore may be the stimulus that triggers angiogenesis in this setting. The resulting neovessels may then eventually become incorporated into the lesion as they become subject to the abnormal hemodynamics in these lesions. It is not known if VEGF or bFGF are indeed upregulated in response to focal ischemia from a-v shunting, or whether they play a role in arterial recruitment, but these hypotheses should be amenable to testing in experimental models of a-v shunts.

The expression of both angiogenic factors was not markedly different in lesions with recent hemorrhage, or in AVMs which had been embolized prior to excision. Recent hemorrhage does not appear to greatly increase the expression of angiogenic factors in the lesions studied, though one may expect that hemorrhage, which by definition requires vessel wall destruction, may be one mechanism by which bFGF is released from its intra- and extracellular stores (32, 46-7) and becomes available to act. Presurgical embolization certainly alters the hemodynamics in the lesional nidus and may create a relatively hypoxic area surrounding an AVM thereby further stimulate angiogenic factor production. However, in both these theoretical situations, it is possible that the level of upregulation is below the level detectable by the immunohistochemical technique.

VEGF and bFGF also appear to be involved in the proliferation of vessels such as caverns in CMs, and small vessels wthin the surrounding gliotic brain. In these lesions, repeated hemorrhages and hence cellular and basement membrane injury, may release bFGF from its intracellular and intrabasement membrane stores to trigger new vessel formation. On the other hand, though CMs do not exhibit the steal phenomena seen in AVMs, the tendency for these slow flowing lesions (13) to thrombose may create situations in which focal ischemia induce angiogenic factor action. VEGF and bFGF may be syngergistic in their action (106), but in these lesions which are masses of vessels with very little extracellular matrix, VEGF may theoretically be the greater perpetrator of angiogenic activity. Other techniques directed at quantifying the amount of VEGF and bFGF activity may clarify this issue in future studies. For example, in situ hybridization to mRNA of VEGF and bFGF would allow measureable comparisons of growth factor production.

The absence of angiogenic factor staining in some caverns may indicate a more quiescent biologic state in portions of the same lesions. Further studies should more carefully correlate the extent and distribution of expression of these growth factors with more recent lesion behavior. In work already under way at our laboratory, we are examining lesions resected electively without any imaging or clinical evidence of recent hemorrhage (e.g., in cases of chronic epilepsy) in comparison with lesions exhibiting unequivocal gross hemorrhage. Preliminary data appears to suggest greater expression of angiogenic factors in recently active lesions, although this will require confirmation with a greater case number.

B. Extracellular Matrix Proteins

Previous immunofluorescent studies from our laboratory using fresh frozen surgical specimens have identified laminin expression in AVMs but not CMs, and more diffuse and prominent fibronectin in CMs than AVMs (12). Those observations have been replicated in this current study using immunohistochemistry on fixed, deparaffinated specimens. LN was detected only in AVMs; among AVM vessels, LN expression was more prominent in thinner walled larger arteries and veins. Vessels with thicker walls, including the one arterialized vein, did not express LN, despite enzymatic pretreatment. It is possible that LN detectability may have been compromised by tissue processing, a potential source of oversight inherent to the immunohistochemical method. Additional studies on fresh frozen specimens with

predigestion protocols may clarify this issue. Although the gross intensity of immunostaining is here reported as 1+ or 2+, it is important to recognize that staining intensity may reflect other factors besides the amount of target protein present. Factors such as location of the lesion within the tissue specimen, tissue preparation, section thickness, paraffinization, enzyme treatment, and variability of chromagen staining are all potentially influential on staining outcome (78, 87, 92). These factors are responsible for most of the limitations of this study. However, one reassuring observation is the reliable presence of strong LN staining in small vessels in perilesional gliotic tissue. These normal vessels, in essence, serve as intraspecimen controls for LN staining technique.

The basement membrane of vascular beds contain varying amounts of FN and LN (22, 77-8, 86-7, 106). Under hemodynamic stress, endothelial cells produce increased amounts of basement membrane proteins (106). These proteins help to maintain structural integrity of the vessel wall by anchoring ECs to the underlying extracellular matrix and adjacent layers of vascular smooth muscle and internal elastic lamina (22, 87, 91). In addition, FN and LN serve a separate and equally important role as the adhesion proteins in a migratory substratum for proliferating ECs. Unlike LN, which is the main component of basal laminae, FN is found thoughout the ECM. Alterations in these proteins may be involved in changes in homeostasis, permeability, and response to injury.

LN is solely expressed in more mature vessels and may contribute to the resilience of these vessels against hemorrhage (22, 87, 106). In the developing embryo, BM proteins may serve a structural function in this way. However, in addition, their ability to influence endothelial cell activity illustrates a signalling function via environmental cues that cause endothelial cell differentiation, proliferation, and spatial organization of vessels in region-specific patterns (21-2, 28, 77). During vasculogenesis and angiogenesis, LN and FN are expressed, or incorporated, into developing vessels at distinct stages (22, 86-7). In general, immature vessels are associated with nonadherent, proliferating endothelium in a FN-rich matrix that is deficient in LN expression. More mature vessels with adherent, stable (nonproliferating) endothelium express LN more consistently and contain scant FN in their matrix. The difference in LN and FN expression in AVMs and CMs may reflect the pathophysiological differences in the biological behavior of these two lesion types. CMs appear to consist of more immature proliferating vessels in a FN-rich matrix

devoid of LN. In contrast, AVMs appear to be composed of morphologically more mature vessels, albeit in an activated state (as suggested by the expression of angiogenic factors) and express more LN.

Both AVMs and CMs stained consistently with CoIV and ASMA. In AVMs, the expression was similar to that seen in normal vessels, with layers of CoIV in the subendothelium and the outer media, and ASMA in the subendothelium and in myocytes and fibrocytes outlining the medial layer. In CMs, both proteins appear to be prominent in the subendothelial layer. CoIV was otherwise very faint (absent altogether in 2/4 cases) in the intercavernous matrix, without the linear band-like expression seen in AVMs. ASMA staining in CMs was also limited to the vessel wall, lacking staining in the media layers such as seen in the AVMs. Other researchers have observed that collagen and ASMA in proliferating blood vessels appear less organized in their histoarchitecture than in more mature vessels (77, 86). The presence of ASMA positivity in CMs is noteworthy, given the absence of morphological smooth muscle layers in the vessel walls of these lesions. Another recent study has identified ASMA expression in ECs and pericytes of infantile and childhood hemangiomas in the involuting phase, though these also lack clearly differentiated myocytes (89). Pericytes, which have a contractile function, form a discontinuous layer from the terminal arteriole to the post-capillary venule, often lying within the basement membrane of EC (Forbes et al, 1977). Like VSMC, pericytes do contain ASMA (Skalli et al., 1989) in their microfilament bundles. Though embryonic data suggest that ECs recruit and organize VSMC from local mesenchymal cells (26), new resarch suggests that ECs may be able to give rise to pericytes and VSMC under certain conditions (108).

C. Hypotheses for AVM & CM Pathogenesis

These observations presented in this study are consistent with activation of the angiogenic process in both AVMs and CMs. This is the first investigation of VEGF and bFGF expression in human vascular malformations of the central nervous system. The precise role of angiogenesis and angiogenic factors in the various lesions has yet to be determined. In AVMs, we postulate that the lesions are a result of dysvasculogenesis during early embryologic development. These lesions have arterial and venous components but completely lack the normal intervening capillary bed, giving rise to a high flow a-v shunt. The expression of these growth factors reflects activation by the high flow state, which produces the

hemodynamic stress capable of inducing morphological changes on both sides of the AVM. In the arterial side, medial hypertrophy and abnormal reduplication of the internal elastic lamina are anatomical changes related to the abnormal hemodynamics; on the venous side, abnormal VSMC hypertrophy appropriately called arterialization is seen. Both of these features strongly suggest the role of bFGF. Focal ischemia (105) in the setting of a-v shunting may induce production of angiogenic factors, which may, in turn, trigger local angiogenesis and facilitate the recruitment of arterial feeding vessels. In a cooperative fashion, the role of VEGF may be also important in AVM angiogenesis activity, but also as a growth factor influential in the endothelium maintenance in face of the high shear stress present in these lesioms.

In CMs, it is likely that genetic predisposition or other factors result in ongoing dysangiogenesis in a FN-rich matrix that results in clusters of immature vessels. These abnormal vessels then hemorrhage due to structural defects in the vessel wall and/or thrombosis resulting from sluggish flow. "Reactive" angiogenic response in the setting of microhemorrhage may lead to further proliferation of abnormal vessels that coalesce into cavernous vessels, thereby creating an enlarging malformation. This is consistent with the frequent presence of capillary malformations in the periphery of CMs. In these lesions, there are marked vessel structures with scant intervening interstitial tissue, which begs the question of whether VEGF may be the more important growth factor in CM dysangiogenesis.

In addition to acting directly on the EC, angiogenic factors may function in angiogenesis by altering tissue dynamics, especially cell-cell and cell-ECM interactions (26). It is tempting to speculate whether the endothelium in CMs is transformed to upregulate its secretion of FN, which in itself helps to create an environment that is conducive for migration and can promote cellular proliferation over differentiation. Further studies to identify the genetic defect in CM, as well as experiments on endothelial cells cultured from both lesion types will be instrumental in further elucidating the roles that angiogenic factors play in lesion biology. An attractive theory is that bFGF's greater function is in areas where there exists a need for connective tissue proliferation along with endothelial mitogenesis (e.g. high stress, AVM arterialized veins) and VEGF is the more influential stimulus in areas where vessel proliferation is seen without excess ECM production (e.g. CMs). With the immunohistochemical technique, quantitative comparisons of the amount of growth factor expression are unwise. Future studies using cultured cells may help to clarify this issue.

D. Present & Future Studies

Recently, there has been some interesting research linking nitric oxide synthase (NOS) and angiogenesis. Nitric oxide (NO) is a vasodilator, formerly known as endothelial derived relaxing factor (EDRF), that is produced by ECs. Both constitutive and inducible NOS systems are found throughout mammalian tissues (110). Loss of EC-derived NO is thought to be one reason why a denuded vessel constricts while undergoing re-endothelialization (27). It appears that release of this relaxing factor can be stimulated by shear stress imposed by flow on the luminal surface of the endothelial cells (111). This vasodilatory action is advantageous in any situation where new vessels are being formed, both physiological and pathological angiogenesis.(112). In support of a pro-angiogenic influence of NOS include studies showing endothlelial NOS association with EC tubule formation (Sessa & Desai, 1994); in vitro NOS inhibition selectively reduces flow in tumor vessels (112); VEGF-induced arterial relaxation (113) and EC locomotion (114) are both dependent on NO; NO mediates cytotoxic release of bFGF from VSMC (115) and bFGF increases NOS in bovine EC (116); in vitro and in vivo demonstration of angiogenesis that is enhanced by substance P (117); and requirement for NO in monocyte-induced angiogenesis (118). Contrary to these, however, is literature demonstrating that NO actually depresses angiogenesis (124-5), possibly as a mediator for other factors such as VEGF and prostaglandin E_1 (119, 126-7). With every new insight into the basic molecular mechanisms, our understanding of the angiogenic process and its various stimulatory and inhibitory loops becomes more complicated. Perhaps similar to the biphasic effect that TGF-b 1 appears to have on VEGF or bFGF potentiated ECs depending on the its concentration (120-1), NOS and other cytokines may be more "contextual" in their angiogeneic activity than can yet be understood (122-3). The presence and concentration of other cytokines and other events in the matrix milieu are important factors.

This thesis was undertaken to explore the possible role that angiogenic growth factors, in conjunction with extracellular matrix proteins, may play in the genesis and biological behavior of vascular malformations in the central nervous system. Obvious limitations of this study are inherent to the immunohistochemical technique as stated above (page 44). Its usefulness as a tool in detecting the

presence of specific antigens is qualitative, not quantitative; therefore the results are fairly descriptive and not subject to quantitative analysis in a strict fashion.

Several lines of investigation would be helpful in furthering the work done in this study, and answering many of the questions raised. More extensive correlation is needed between the angiogenic factor expression and recent lesion behavior, including a dissection of various compartments of the same lesion. This approach has already identified a distinctive temporal expression of specific molecular markers in peripheral hemangioma of infancy and childhood during the various growth phases of the lesions (50). A similar molecular dissection of nervous system vascular malformations may identify a differential expression of angiogenic factors and select matrix proteins in the nidus, arterial and venous components of AVMs, and in hemorrhagic versus fibrotic components of CMs as seen microscopically at the time of resection.

Use of the proliferating cell nuclear antigen (PCNA) would be of interest to detect active endothelial cell S-phase activity and correlate with angiogenic growth factor expression or recent clinical behavior. Given the potential ability of a number of tissues to produce mRNA and bind these angiogenic factors, an assessment of whether VEGF and bFGF receptors are upregulated in these vascular malformations would also be a way of understanding how angiogenesis may be increased in these lesions.

Lines of EC and fibrocyte cultures should be established from resected AVMs and CMs (18). These will allow phenotypic characterization of cell lines and exploration of specific mRNA and protein expression, at baseline and in response to specific factors such as in vitro shear stress and hypoxia. Specific hypotheses including whether shear stress or hypoxia in culture will alter the molecular expression or behavior of these cells could be tested and quantitative analyses made in vitro. The hope is that new knowledge gained in these pursuits will result in effective therapeutic strategies, such as angiogenesis inhibition, aimed at modifying the clinical behavior of these lesions.

Finally, characterization of the time course of matrix protein alterations in relation to angiogenic factor expression is needed, and may be explored in experimental arterialized venous beds and in animal models of hemorrhagic proliferative angioma. Intensive studies are underway at a number of labs, including our own, to characterize the genetic substrate of familial vascular malformations (15). Identification of specific

gene products will allow another dimension of experimental studies aiming to link the respective molecules with the phenotypic and clinical manifestations in the lesions.

V. REFERENCES

Cerebrovascular Malformations

1. Awad IA, Robinson JR, Mohanty S, Estes ML. Mixed vascular malformations of the brain: clinical and pathogenetic considerations. Neurosurgery 33: 179-88, 1993.
2. Robinson JRR, Awad IA, Masaryk TJ, Estes ML. Pathological heterogeneity of angiographically occult vascular malformations of the brain. Neurosurgery 33: 547-54, 1993.
3. Kraemer DL, Awad IA. Vascular malformations and epilepsy: clinical considerations and basic mechanisms. (Review). Epilepsia 35 Suppl 6: S30- 43, 1994.
4. Kondziolka D, Lunsford LD, Kestle JR. The natural history of cerebral cavernous malformations. JNUS 85: 820-4, 1995.
5. DeReuck J, Dela Meilleure G, Boon P, Decoo D, Strijckmans K, Goethals P, Lemahien I. Comparison of cerebral hemodynamic and oxygen metabolic changes due to cavernous angioma and arteriovenous malformation of the brain. A positron emission tomography study. Acta Neurologica Belgica 94 (4): 239- 44, 1994.
6. Wallinsky R, Terbrugge K, Montanera W, Mikulis D, Wallace MC. Venous congestion: an MRI finding in dural AVM with cortical venous drainage. AJNR 15 (8): 1501- 7, 1994.
7. Miyachi S, Negoro M, Handa T, Sugita K. Contribution of meningeal arteries to cerebral arteriovenous malformations. Neuroradiol 35 (3): 205- 9, 1993.
8. Robinson JR, Awad IA, Little JR. Natural history of the cavernous angioma. J Neurosurg 75 (5): 709-14, 1991.
9. Aiba T, Tanaka R, Koike T, Kameyama S, Takeda N, Komata T. Natural history of intracranial cavernous malformations. JNUS 83: 56-9, 1995.
10. Rigamonti D, Johnson PC, Spetzler RF, Hadley MN, Drayer BP. Cavernous malformations and capillary telangiectasia: a spectrum within a single pathological entitiy. Neurosurg 28 (1): 60- 4, 1991.
11. Robinson JR Jr, Awad IA, Magdinec M, Paranandi L. Factors predisposing to clinical disability in patients with cavernous malformations of the brain. Neurosurg 32 (5): 730- 5m 1993.
12. Robinson JRR, Awad IA, Zhou P, Barna BP, Estes ML. Expression of basement membrane and endothelial cell adhesion molecules in vascular malformations of the brain: Preliminary observations and working hypothesis. Neurol Res 17: 49-58, 1995.
13. Little JR, Awad IA, Jones SC, Ebrahim ZY. Vascular pressures and cortical blood flow in cavernous angioma of the brain. JNUS 73: 555-9, 1990.
14. Maraire JN, Awad IA. Intracranial Cavernous Malformations: lesion behavior and management strategies. Neurosurgery 37: 591-605, 1995.
15. Gunel M, Awad IA, Anson J, Lifton R. Mapping a gene causing cerebral cavernous malformation to 7q11.2-q21. PNAS USA 92 (14): 6620- 4, 1995.
16. Dubovsky J, Zabramski JM, Kurth J, Spetzler RF, Rich SS, Orr HT, Weber JL. A gene responsible for cavernous malformations of brain maps to chromosome 7q. Human Molec Genetics 4 (3): 453- 8, 1995.
17. Zabramski JM, Wascher TM, Spetzler FR, Johnson B, Goldfinos J, Drayer BP, Brown B, Rigamonti D, Brown G. The natural history of familial cavernous malformations: results of an ongoin study. J Neurosurg 80 (3): 922- 32, 1944.
18. Comair Y, Rhoten P, de la Motte C, Awad I, DiCorleto P. Isolation and characterization of endothelial cells in vascular malformations. Presented at the 62nd Annual Meeting of the American Association of Neurological Surgeons, San Diego, April, 1994.
19. Okazaki H. Fundamentals of Neuropathology. Morphologic Basis of Neurologic Disorders. Second edition. (Igaku- Shoin Medical Publishers, Inc, New York, 1989).

Angiogenesis & Angiogenic Factors

20. Reagan FP. Vascularization phenomena in fragments of embryonic bodies completely isolated from yolk sac blastoderm. Anat Rec 9: 329-341, 1915.
21. Coffin JD, Poole TJ. Embryonic vascular development: immunohistochemical identification of the origin and subsequent morphogenesis of the major vessel primordia in quail embryos. Development 102: 735-48, 1988.

22. Risau W, Lemmon V. Changes in the vascular extracellular matrix during embryonic vasculogenesis and angiogenesis. Dev Biol 125: 441-50, 1988.
23. Cliff WJ. Observations on healing tissue: a combined light and electron microscopic investigation. Philos Trans R Soc London Ser B 246: 305-24, 1963.
24. Clark RAF, DellaPelle P, Manseau E, Lanegan JM, Dvorak HF, Colvin RB. Blood vessel fibronectin increases in conjunction with endothelial cell proliferation and capillary ingrowth during wound healing. J Invest Dermatol 79: 269-74, 1982.
25. Folkman J, Klagsburn M. Angiogenic factors. (Review). Science 235: 442-7, 1987.
26. Schwartz SM, Heimark RL, Majesky MW. Developmental mechanisms underlying pathology of arteries. Physiological Rev 70 (4): 1177-1209, 1990.
27. Bobik A, Campbell JH. Vascular derived growth factors: cell biology, pathophysiology, and pharmacology. (Review). Pharm Rev 45: 1-42, 1993.
28. Keep RF, Jones HC. Cortical microvessels during brain development: a morphometric study in the rat. Microvasc Res 40: 412-26, 1990.

Basic Fibroblast Growth Factor

29. Gospodarowicz D, Ferrara N, Schweigerer L, Neufeld G. Structural characterization and biological functions of fibroblast growth factor. (Review). Endo Rev 8: 95-114, 1987.
30. Risau W. Angiogenic growth factors. (Review). Prog Growth Factor Res 2: 71-9, 1990.
31. Moscatelli D, Presta M, Rifkin DB. Purification of a factor from human placenta that stimulates capillary endothelial cell protease production, DNA synthesis, and migration. PNAS USA 83: 2091-5, 1986.
32. Schweigerer L, Neufeld G, Friedman J, Abraham JA, Fiddes JC, Gospodarowicz D. Capillary endothelial cells express basic fibroblast growth factor, a mitogen that promotes their own growth. Nature 325: 257-9, 1987.
33. Gospodarowicz D, Ferrara N, Haaparanta T, Neufeld G. Basic fibroblast growth factor: expression in cultured bovine vascular smooth muscle cells. Eur J Cell Biol 46: 144-51, 1988.
34. Henke C, Marinelli W, Jessurun J, Fox J, Harms D, Peterson M, Chiang L, Doran P. Macrophage production of basic fibroblast growth factor in the fibroproliferative disorder of alveolar fibrosis after lung injury. Am J Path 143 (4): 1189- 99, 1993.
35. Montesano R, Vassali JD, Baird A, Guillemin R, Orci L. Basic FGF induces angiogenesis in vitro. PNAS USA 83: 7297- 7301, 1986.
36. Rifkin DB, Moscatelli D. Recent developments in the cell biology of basic fibroblast growth factor. (Review). J Cell Biol 109: 1-6, 1989.
37. Ferrara N, Ousley F, Gospodarowicz. Bovine brain astrocytes express basic fibroblast growth factor, a neurotropic and angiogenic mitogen. Brain Res 462: 223-232, 1988.
38. Sato Y, Murphy PR, Sato R, Friesen HG. Fibroblast growth factor release by bovine endothelial cells and human astrocytoma cells in culture is density dependent. Mol Endocrine 3: 744-8, 1989.
39. Paulus W, Grothe C, Sensenbrenner M, Janet T, Baur I, Graf M, Roggendorf W. Localization of basic fibroblast growth factor, a mitogen and angiogenic factor, in human brain tumors. Acta Neuropathol 79: 418-23, 1990.
40. Takahashi JA, Mori H, Fukumoto M, Igarashi K, Jaye M, Oda Y, Kikuchi H, Hatanaka M. Gene epxression of fibroblast growth factors in human gliomas and meningiomas: demonstration of cellular source of basic fibroblast growth factor mRNA and peptide in tumor tissue. PNAS USA 87: 5710-4, 1990.
41. Zagzag D, Miller DC, Sato Y. Immunohistochemical localization of bFGF in astrocytomas. Cancer Res 50: 7393-8, 1990.
42. Abraham JA, Mergia A, Whang JL, Tumolo A, Friedman J, Hjerrild KA, Gospodarowicz D, Fiddes JC. Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. Science 233: 545- 8, 1986.
43. Neufeld G, Gospodarowicz D. Basic and acidic fibroblast growth factors interact with the same cell suface receptors. J Biol Chem 261: 5631- 7, 1986.
44. Moenner M, Chevallier B, Badet J, Barritault D. Evidence and characterization of the receptor to eye-derived growth factor I, the retinal form of basic fibroblast growth factor, on bovine epithelial lens cells. PNAS USA 83: 5024- 8, 1986.
45. Olwin BB, Hauschka SD. Identification of the fibroblast growth factor receptor of Swiss 3T3 cells and mouse skeletal muscle myoblasts. Biochem 25: 3487- 92, 1986.

46. Folkman J, Klagsbrun M, Sasse J, Wadzinski M. A heparin-binding angiogenic protein-- bFGF-- is stored within the basement membrane. Am J Path 130 (2): 393-400, 1988.
47. Vlodasky I, Fuks Z, Ishai MR, Bashkin P, Levi E, Korner G, BarShavit R, Klagsbrun M. Extracellular matrix-resident basic fibroblast growth factor: implication for the control of angiogenesis. (Review). J Cell Biochem 45: 167-76, 1991.
48. Tsuboi R, Sato Y, Rifkin DB. Correlation of cell migration, cell invasion, receptor number, proteinase production, and basic fibroblast growth factor levels in endothelial cells. J Cell Biol 110: 511-7, 1990.

Vascular Endothelial Growth Factor (VEGF)/ Vascular Permeability Factor (VPF)

49. Senger DR, Perruzzi CA, Feder J, Dvorak HF. A highly conserved vascular permeability factor secreted by a variety of human and rodent tumor cells lines. Cancer Res 46: 5629-32, 1986.
50. Ferrara N, Henzel WJ. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun 161: 851-8, 1989.
51. Gospodarowicz D, Abraham JA, Schilling J. Isolation and characterization of a vascular endothelial cell mitogen produced by pituitary-derived folliculo stellate cells. PNAS USA 86: 7311-5, 1989.
52. Senger DR, Connolly DT, VanDeWater L, Feder J, Dvorak HF. Purification and NH2-terminal amino acid sequence of guinea pig tumor-secreted vascular permeability factor. Cancer Res 50: 1774-8, 1990.
53. Connolly DT, Heuvelman DM, Nelson R, Olander JV, Eppley BL, Delfino JJ, Siegel NR, Leimgruber RM, Feder J. Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. J Clin Invest 84: 1470-8, 1989.
54. Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT. Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science 246: 1309-12, 1989.
55. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246: 1306-9, 1989.
56. Bruce JN, Criscuolo GR, Merrill MJ, Moquin RR, Blacklock JB, Oldfield EH. Vascular permeability induced by protein product of malignant brain tumors: inhibition by dexamethasone. JNUS 67: 880-4, 1987.
57. Criscuolo GR. The genesis of peritumoral vasogenic brain edema and tumor cysts: a hypothetical role for tumor derived vascular permeability factor. (Review). Yale J Biol Med 66: 277-314, 1993.
58. Strugar J, Rothbart D, Harrington W, Criscuolo GR. Vascular permeability factor in brain metastases: correlation with vasogenic brain edema and tumor angiogenesis. JNUS 81: 560-6, 1994.
59. Strugar J, Criscuolo GR, Rothbart D, Harrington W. Vascular endothelial growth/permeability factor expression in human glioma specimens: correlation with vasogenic brain edema and tumor-associated cysts. JNUS 83: 682-9, 1995.
60. Berkman RA, Merrill MJ, Reinhold WC, Monacci WT, Saxena A, Clark W, Robertson JT, Ali IU, Oldfield EH. Expression of the vascular permeability factor/vascular endothelial growth factor gene in central nervous system neoplasms. J Clin Invest 91: 153-9, 1993.
61. Criscuolo GR, Merrill MJ, Oldfield EH. Further characterization of malignant glioma-derived vascular permeability factor. JNUS 69: 254-62, 1988.
62. Myoken Y, Kayada Y, Okamoto T, Kan M, Sato GH, Sato JD. Vascular endothelial cell growth factor (VEGF) produced by A-431 human epidermoid carcinoma cells and identification of VEGF membrane binding sites. PNAS USA 88: 5819-23, 1991.
63. Jakeman LB, Winer J, Bennett GL, Altar CA, Ferrara N. Binding sites for vascular endothelial growth factor are localized on endothelial cells in adult rat tissues. J Clin Invest 89: 244-53, 1992.
64. Criscuolo GR, Leikes PI, Rotrosen D, Oldfield EH. Cytosolic calcium changes in endothelial cells induced by a protein product of human gliomas containing vascular permeability factor activity. J Neurosurg 71: 884- 91, 1989.
65. Drake CJ, Little CD. Exogenous vascular endothelial growth factor induces malformed and hyperperfused vessels during embryonic neovascularization. PNAS USA 92 (17) : 7657-7661, 1995.
66. Peters KG, deVries C, Williams LT. Vascular endothelial growth factor receptor expression during embryogenesis and tissue repair suggests a role in endothelial differentiation and blood vessel growth. PNAS USA 90 (19): 8915- 9, 1993.
67. deVries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science 255: 989-91, 1992.

68. Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC, Abraham JA. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. J Biol Chem 266: 11947- 54, 1991.
69. Shalaby F, Rossant J, Yamaguchi T. Failure of blood-island formation and vasculogenesis in Flk-1 deficient mice. Nature 376: 62-6, 1995.
70. Fong GH, Rossant J, Gertsenstein M. Role of Flt-1-R tyrosine kinase in regulating the assembly of vascular endothelium. Nature 376: 66-70, 1995.
71. Sato TN, Tozawa Y, Dentsch U. Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. Nature 376: 70-4, 1995.
72. Dvorak HF, Brown LF, Detmar M, Dvorak AM. Vascular Permeability Factor/ Vascular endothelial growth factor, microvascular permeability, and angiogenesis. (Review). Am J Path 146 (5): 1029- 1039, 1995.
73. Clauss M, Gerlach M, Gerlach H, Brett J, Wang F, Familetti PC, Pan YC, Olander JV, Connolly DT, Stern D. Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration. J Exp Med 172: 1535-45, 1990.

Extracellular Matrix Proteins

74. Wheater PR, Burkitt HG, Daniels VG, Eds. Functional Histology : A Text and Color Atlas. Second edition. (Churchill Livingstone, 1987)
75. Rutka JT, Apodaca G, Stern R, Rosenblum M. The extracellular matrix of the central and peripheral nervous stystems: structure and function. J Neurosurg 69: 155-70, 1988.
76. Ushiwata I, Ushiki T. Cytoarchitecture of the smooth muscles and pericytes of rat cerebral blood vessels: a scanning electron microscope study. J Neurosurg 73: 82- 90, 1990.
77. Navaratnam V. Organisation and reorganisation of blood vessels in embryonic development. (Review). Eye 5: 147- 50, 1991.
78. Garbisa S, Negro A. Macromolecular organization and functional architecture of basement membranes. Appl Path 2: 217-22, 1984.
79. Grant DS, Kleinman HK, Martin GR. The role of basement membranes in vascular development. Ann NY Acad Sci 588: 61- 72, 1990.
80. Furcht LT. Critical factors controlling angiogenesis: cell products, cell matrix, and growth factors. Lab Invest 55: 505-9, 1986.
81. Madri JA, Pratt BM, Tucker AM. Phenotypic modulation of endothelial cells by transforming growth factor-b depends upon the composition and organization of the extracellular matrix. J Cell Biol 106: 1365- 73, 1988.
82. Herebst TJ, McCarthy JB, Tsilibary EC, Furcht LT. Differential effects of laminin, intact type IV collagen, and specific domains of the type IV collagen on endothelial cell adhesion and migration. J Cell Biol 106: 1365- 73, 1988.
83. KubotaYH, Kleinman HK, Martin GR, Lawley TJ. Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. J Cell Biol 107: 1589- 98, 1988.
84. Madri JA, Williams SK, Wyatt T, Mezzio C. Capillary endothelial cell cultures: phenotypic modulation by matrix components. J Cell Biol 97: 153- 65, 1983.
85. Fukuda K, Koshihara Y, Oda H, Ohyama M, Ooyama T. Type V collagen selectively inhibits human endothelial cell proliferation. Biochem Biophys Res Comm 151: 1060- 8, 1988.
86. Liakka A, Apaja SM, Karttunen T, Autio HH. Distribution of laminin and types IV and III collagen in fetal, infant, and adult human spleens. Cell Tissue Res 263: 245- 52, 1991.
87. Krum JM, More NS, Rosenstein JM. Brain angiogenesis: variations in vascular basement membrane glycoprotein immunoreactivity. Exp Neurol 111: 152- 65, 1991.
88. Sawada T, Nakamura M, Sakurai I. An immunohistochemical study of neurovasculature in human brain tumors. Acta Pathol Jpn 38 (6): 713- 21, 1988.

Implications for Vascular Disorders in the CNS

89. Takahashi K, Mulliken JB, Kozakewich HPW. Cellular markers that distinguish the phases of hemangioma during infancy and childhood. J Clin Invest 93: 2357- 54, 1994.

90. McAllister KA, Grogg KM, Johnson DW, Gallione CJ, Baldwin MA, Jackson CE, Helmbold EA, Markel DS, McKinnon WC, Murrell J, McCormick MK, Pericak-Vance MA, Heutnik P, Costra BA, Haitjema T, Westerman CJJ, Porteous ME, Guttmacher AE, Letarte M, Marchuk DA. Endoglin, a TGF-b binding protein of ECs, is the gene for hereditary haemorrhagic telangiectasia type 1. Nature Genetics 8: 345- 51, 1994.
91. Merei FT, Gallyas F, Horvath Z. Elastic elements in the media and adventitia of human intracranial extracerebral arteries. Stroke 11: 329- 335, 1990.
92. Mauro A, Bertolotto A, Germano I, Giaccone G, Giordana MT, Migheli A, Schiffer D. Collagenase in the immunohistochemical demonstration of laminin, fibronectin, and factor VIII/ RAg in nervous tissue after fixation. Histochemistry 80: 157- 63, 1984.
93. Baron-Van Evercooren A, Kleinman HK, Ohno S, Marangos P, Schwartz JP, Dubois-Dalcq ME. Nerve growth factor, laminin, and fibronectin promote neurite growth in human fetal sensory ganglia cultures. J Neurosci Res 8: 179- 93, 1982.
94. McGarvey ML, Baron-Van Evercooren A, Kleinman HK, Dubois-Dalcq. Synthesis and effects of basement membrane components in cultured rat Schwann cells. Dev Biol 105: 18- 28, 1984.
95. Liesi P, Dahl D, Vaheri A. Laminin is produced by early rat astrocytes in primary culture. J Cell Biol 96: 920- 4, 1983.
96. Liesi P, Kirkwood T, Vaheri A. Fibronectin is expressed by astrocytes cultured from embryonic and early postnatal rat brain. Exp Cell Res 163: 175- 85, 1986.
97. Rutka JT, Giblin JR, Dougherty DV, McCulloch JR, DeArmond SJ, Rosenblum ML. An ultrastructural and immunocytochemical analysis of leptomeningeal and meningioma cultures. J Neuropathol Exp 45: 285- 303, 1986.
98. Hoshimaru M, Takahashi JA, Kikuchi H, Nagata I, Hatanaka M. Possible roles of basic fibroblast growth factor in the pathogenesis of moyamoya disease: an immunohistochemical study. J Neurosurg 75: 267- 70, 1991.

Objectives&Hypotheses

99. Skirgaudas M, Awad IA, Kim J, Rothbart D, Criscuolo G. Expression of angiogenesis factors and selected vascular wall matrix proteins in intracranial saccular aneurysms. Neurosurgery (in press).

Immunohistochemistry

100. Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 29: 577-80, 1981.
101. Henke C, Marinelli W, Jessurum J, Fox J, Harms D, Peterson M, Chiang L, Doran P. Macrophage production of basic fibroblast growth factor in the fibroproliferative disorder of alveolar fibrosis after lung injury. Am J Path 143: 1189- 99, 1993.
102. Sternberger LA, Sternberger NH. The unlabeled antibody method: comparison of peroxidase-antiperoxidase with avidin-biotin complex by a new method of quantification. J Histochem Cytochem 34: 599- 605, 1986.
103. Kirkpatrick P, D'Ardenne AJ. Effects of fixation and enzymatic digestion on the immunohistochemical demonstration of laminin and fibronectin in paraffin embedded tissue. J Clin Path 37: 639- 44, 1984.

Discussion

104. Herman JM, Spetzler FR, Bederson JB. Genesis of a dural AVM in a rat model. J Neurosurg 83: 539- 45, 1995.
105. Schweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 359: 843-5, 1992.
106. Pepper MS, Ferrara N, Orci L, Montesano R. Potent synergism between VEGF and bFGF in induction angiogenesis in vitro. Biochem Biophys Res Comm 189 (2): 824-31, 1992.
107. Kittelberger R, Davis PF, Stehbens WE. Distribution of type IV collagen, laminin, nidogen and fibronectin in the hemodynamically stressed vascular wall. Histo Histopath 5: 161- 7, 1990

108. Madri JA, Bell L, Marx M, Merwin JR, Basson C, Prinz C. The effects of soluble factors and extracellular matrix components on vascular cell behavior in vitro and in vivo: models of de-endothelialization and repair. J Cellular Biochem 45: 123-30, 1991.

Nitric Oxide Synthase and Angiogenesis

110. Knowles RG, Moncada S. Nitric oxide synthases in mammals. Biochem J 298 (pt 2): 249-58, 1994.
111. Vanhoutte PM, Rubanyi GM, Miller VM, Houston DS. Modulation of vascular smooth muscle contraction by the endothelium. Ann Rev Physiol 48: 307-20, 1986.
112. Andrade SP, Hart IR, Piper PJ. Inhibitors of nitric oxide synthase selectively reduce flow in tumor-associated neovasculature. Br J Pharmacol 107: 1092- 5, 1992.
113. Ku DD, Zaleski JK, Liu S, Brock TA. Vascular endothelial growth factor induces EDRF-dependent relaxation in coronary arteries. Am J Physiol 265 (2 Pt 2): H586-92, 1993.
114. Noiri E, Peresleni T, Testa J, Quigly J, Goligorsky MS. Vascular endothelial growth factor (VEGF) stimulated endothelial cell (EC) locomotion: absolute requirement for nitric oxide (NO). (Abstract).
115. Fukuo K, Inoue T, Morimoto S, Nakahashi T, Yasuda O, Kitano S, Sasada R, Ogihara T. Nitric oxide mediates cytotoxity and bFGF release in cultured vascular smooth muscle cells. A possible mechanism of neovascularization in atherosclerotic plaques. J Clin Invest 95(2): 669-76, 1995.
116. Kostyk SK, Kourembanas S, Wheeler. bfGF incr NOS in bovine EC. Am J Physiol 269: H1583-9, 1995.
117. Ziche M, Morbidelli L, Masini E, Amerini S, Granger HJ, Maggi CA, Geppetti P, Ledda F. Nitric oxide mediates angiogenesis in vivo and endothelial cell growth and migration in vitro promoted by substance P. J Clin Invest 94: 2036- 44, 1994.
118. Leibovich SJ, Polverini PJ, Fong TW, Harlow LA, Koch AE. Production of angiogenic activity by human monocytes requires an L-arginine/ nitric oxide-synthase-dependent effector mechanism. PNAS USA 91: 4190-4, 1994.
119. Finder J, Stark WW, Nakayama DK, Geller D, Wasserloos K, Pitt BR, Davies P. TGF-b regulates production of NO in pulmonary artery smooth muscle cells by inhibiting expression of NOS. Am J Physiol 268: L862-7, 1995.
120. Pepper MS, Vassali JD, Orci L, Montesano R. Biphasic effect of transforming growth factor beta 1 on in vitro angiogenesis. Exp Cell Res 204 (2): 356- 63, 1993.
121. Lawrence DA. Transforming growth factor-b: an overview. Kidney International 47 (Suppl 49): S19- S23, 1995.
122. Jenkins DC, Charles IG, Thomsen LL, Moss DW, Holmes LS, Baylis SA, Rhodes P, Westmore K, Emson PC, Moncada S. Roles of nitric oxide in tumor growth. PNAS USA 92: 4392-4396, 1995.
123. McQuillian LP, Leung GK, Marsden PA, Kostyk SK, Kourembanas S. Hypoxia inhibits eNOS expression via transcriptional and posttranscriptional mechanisms. Am J Phys 267 (5Pt2): H1921-7, 1994.
124. Pipili-Synetos E, Sakkoula E, Maragoudakis ME. Nitric oxide in involved in the regulation of angiogenesis. Br J Pharmacol 108: 855-7, 1993.
125. Pipili-Synetos E, Sakkoula E, Haralabopoulos G, Andriopoulou P, Peristeris P, Maragoudakis ME. Evidence that nitric oxide is an endogenous antiangiogenic mediator. Br J Pharmacol 111: 894-902, 1994.
127. Ziche M, Morbedilli L, Parenti A, Ledda F. Nitric oxide modulates angiogenesis elicited by prostaglandin E1 in rabbit cornea. Adv Prostaglandin Thromboxane & Leukotriene Res 23: 495-7, 1995.

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